NITROGEN METABOLISM IN THE AFRICAN LUNGFISH, *PROTOPTERUS ANNECTENS* DURING AESTIVATION: AIR VERSUS MUD, AND NORMOXIA VERSUS HYPOXIA

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A THESIS SUMITTED

FOR THE DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

NATIONAL UNIVERSITY OF SINGAPORE

2009

ACKNOWLEDGEMENTS

My success, my achievements, my honours, if I ever have now or future, are all yours, Prof Ip. A mere thank you is not good enough for what you have done for me. To show my gratitude to you, I will carry on thinking, practising, reflecting, and learning what you taught.

Zillion thanks to Mdm for your kindness and patience to me all these years. You are a great friend and advisor, and my brain un-blocker at times, really.

Billion thanks to all my friends. Your presence put a smile on my face.

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SUMMARY

This study aimed to examine nitrogen metabolism in the African lungfish, *Protopterus annectens*, during aestivation in air or mud and in normoxia or in hypoxia. Results obtained indicate that *P. annectens* was ureogenic, and possessed carbamoyl phosphate synthetase III (CPS III) in the liver. Fish aestivating in air depended more on an increased urea synthesis than a decreased ammonia production to avoid ammonia toxicity, and vice versa for fish aestivating in mud which could be responding to a combination of aestivation and hypoxia. Overall, results obtained from this study indicate the importance of deifining hte hypoix astatus of the aestivating lungfish in future studies. Additionally, efforts should be made to elucidate mechanisms involved in the induction and the arousal phase during which increased protein synthetsis and degradation may occure simultaneously for reconstruction and reorganiszation of cells and tissue which could be important facet of the aestivation process.

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1. Abstract

This study aimed to examine nitrogen metabolism in the African lungfish, *Protopterus annectens*, during aestivation in air or mud and in normoxia or in hypoxia. Results obtained indicate that *P. annectens* was ureogenic; it possessed carbamoyl phosphate synthetase III (CPS III), and not CPS I, in the liver as reported previously. Fish aestivating in air depended more on an increased urea synthesis than a decreased ammonia production during the induction and early maintenance phases of aestivation (first 12 days), but decreased ammonia production was a more important adaptation during the maintenance phase (46 days). By contrast, fish aestivating in mud for 46 days did not accumulate urea due to a profound suppression of ammonia production. Since fish aestivated in mud had relatively low blood pO_2 and muscle ATP content, they could have been exposed to hypoxia, which induced reductions in metabolic rate and ammonia production. Indeed, the rate of urea synthesis increased 2.4-fold, with only a 12% decrease in the rate of N production in the fish during 12 days of aestivation in normoxia, but the rate of ammonia production in the fish aestivating in hypoxia (2% O_2 in N_2) decreased by 58%, with no increase in the rate of urea synthesis. A reduction in the dependency on increased urea synthesis to detoxify ammonia, which is energy intensive by reducing ammonia production, would conserve cellular energy during aestivation in hypoxia. Indeed, there were significant increases in glutamate concentrations in tissues of fish aestivating in hypoxia, which indicates decreases in its degradation and/or transamination. Furthermore, there were significant increases in the hepatic glutamate dehydrogenase amination activity, the amination/deamination ratio and the dependency of the amination activity on ADP activation in fish on days 6 and 12 in hypoxia, but similar changes occurred only in the normoxic fish on day 12. Therefore, these results confirm that *P. annectens* exhibited different adaptive responses during aestivation in normoxia and in hypoxia. They also indicate that reduction in nitrogen metabolism, and probably metabolic rate, did not occur simply in association with aestivation (in normoxia)

but responded more effectively to a combined effect of aestivation and hypoxia. Results obtained using suppression subtractive hybridization further confirmed the up-regulation of mRNA expression of several genes related to urea synthesis, i.e. *cps*, *ass* and *gs* in fish after 6 days of aestivation in air or in hypoxia. In addition, mRNA expression of several gene clusters were up- or down-regulated during the induction phase of aestivation, and 6 days of aestivation in hypoxia led to up-regulation of genes related to anaerobic energy metabolism, some of which were instead down-regulated in fish aestivated in normoxia for 6 days. Hence, it can be concluded that increased fermentative glycolysis was a response to hypoxia and not intrinsic to the aestivation process. Results obtained from qPCR reveal that mRNA expression of *cps*, *ass*, *gs* and *gdh* were differentially controlled during the induction, maintenance and arousal phases of aestivation in air. There were also subtle differences in mRNA expression of these four genes during the induction phase and early maintenance phase of aestivation in normoxia and in hypoxia. Overall, results obtained from this study indicate the importance of defining the hypoxic status of the aestivating lungfish in future studies. Additionally, efforts should be made to elucidate mechanisms involved in the induction and the arousal phases during which increased protein synthesis and degradation may occur simultaneously for reconstruction and reorganization of cells and tissues which could be important facets of the aestivation process.

2. Overall Introduction

2.1. Aestivation involves fasting, desiccation, high temperature and corporal torpor

Suspended animation has long fascinated scientists because of its great application potentials in fields ranging from medicine to space travel. Animals become inactive during suspended animation. They have absolutely no intake of food and water, and hence produce minimal or no urine and fecal materials for an extended period. They enter into a state of torpor, slowing down the biological time in relation to the clock time. In nature, suspended animation is expressed in adult animals undergoing hibernation or aestivation. Aestivation occurs widely in both vertebrates and invertebrates to survive arid conditions at high temperature, in many cases during summer. Aestivation has been used as a term to describe pulmonate land snails that retract into their shells and remain dormant in the absence of water (Brooks and Storey, 1995; Solomon et al., 1996), sea cucumbers that remain inactive in water at high temperature (Li et al., 1996; Liu et al., 1996; Ji et al., 2008), African lungfishes that remain motionless in a mud cocoon up to three years during drought (Smith, 1930; Fishman et al., 1987; Chew et al., 2004; Loong et al., 2005; 2008a, b), amphibians which make cocoons that encase them for weeks or more than a year during "summer sleep" (Withers and Guppy, 1996; Hudson et al., 2002a, b), and listless state of ground squirrels and cactus mouse at the height of summer heat (Wilz and Heldmaier, 2000). In comparison with hibernation, which occurs in response to cold temperature, aestivation is more intriguing and fascinating because a state of corporal torpor is achieved at high environmental temperature. Conditions that lead to suspended animation have profound effects on nitrogen metabolism and excretion in hibernators and aestivators.

2.2. Corporal torpor with or without metabolic depression

From the behavioral point of view, aestivation could be defined as terrestrial inactivity at high environmental temperature, particularly during dry seasons (Gregory, 1982;

Peterson and Stone, 2000). Ultsch (1989) advanced the all-behavior position, calling aestivation "a non-mobile fossorialism". From the physiological point of view, aestivation has often been associated with metabolic depression (Storey, 2002), because conservation of metabolic fuels has been regarded as an important adaptation during long periods of aestivation without food intake. While this association is clearly present in endothermic mammals during aestivation, it is debatable whether it can be universally applied to aestivating ectothermic animals. For instance, it has been proposed that metabolic depression (Storey and Storey, 1990; Guppy and Withers, 1999) would decrease both urea production and respiratory water loss, in addition to conserving metabolic fuels, in aestivating turtle (Seidel, 1978; Kennett and Christian, 1994; Hailey and Loveridge, 1997). However, whether metabolic depression in turtles is an adaptation to aestivation per se or simply a response to fasting (Rapatz and Musacchia, 1957; Belkin, 1965; Sievert et al., 1988) remains an open question. In fact, the decrease in oxygen consumption in laboratory-aestivating yellow mud turtle *Kinosternon flavescens* is identical to that of fully hydrated turtles that are fasted for an equivalent period (Seidel, 1978; Hailey and Loveridge, 1997). Furthermore, the high body temperature of some aestivating turtles (Kennett and Christian, 1994; Christian et al., 1996) would pose serious constraints to the magnitude of metabolic depression that can be achieved.

For African lungfishes, it has long been accepted that a profound decrease in metabolic rate occurs in association with aestivation in a mud cocoon or an artificial substratum (Smith, 1935; Janssens and Cohen 1968a, b) but without any knowledge on whether aestivation takes place in hypoxia or normoxia. Recently, it was demonstrated that the slender lungfish, *Protopterus dolloi*, aestivating in a completely dried mucus cocoon in air (normoxia) had a respiratory rate comparable to that of control fish immersed in water (Perry et al. 2008; the application of the term "terrestrialization" to these fish was inappropriate; see comments by Loong et al., 2008a and Chapter 2), and the respiratory rate

of fish immersed in water was greatly reduced by aerial hypoxia (Perry et al. 2005a). It is therefore logical to reason that there could be a greater reduction in the metabolic rate of fish aestivating in hypoxia than in normoxia, resulting in a greater suppression in nitrogen metabolism in the former than in the latter. It would mean that metabolic depression in aestivating African lungfish could be triggered by hypoxia and may not be an integral part of aestivation. Hence, it may be more appropriate to regard aestivation as a state of summer corporal torpor with or without metabolic rate reduction, depending on the environmental conditions and the animal species involved. For instance, it can be reasoned that African lungfishes could have lower metabolic rates during aestivation in subterranean mud cocoons (i.e. in hypoxia) as compared with during aestivation in air (i.e. in normoxia). This is an important point because metabolic rate reduction encompasses processes like ammonia production and urea synthesis which are energy dependent and has been conceptually linked in part with suppression of protein synthesis.

2.3. Current issues on excretory nitrogen metabolism and related phenomena in aestivators

2.3.1. Aestivation in normoxia or hypoxia?

It is difficult to interpret information available in the literature on nitrogen metabolism in aestivating animals because over many instances, it is uncertain whether the aestivating animal was being exposed to hypoxia, and if so the degree of hypoxia involved. As a result, it is difficult to analyze phenomena incidental to aestivation independent of hypoxia, but it is important to do so because of the observation made by Perry et al. (2005a) on *P. dolloi*. Aestivation in mud or an artificial substratum may prescribe exposure to hypoxia, and indeed it has been demonstrated that aestivation in mud exerts different effects from aestivation in air on excretory nitrogen metabolism in the swamp eel, *Monopterus albus* (Chew et al., 2005a).

2.3.2. Induction, maintenance and/or arousal?

There was a lack of effort in the past to identify and examine phenomena associated specifically with a certain phase of aestivation, and hence it becomes difficult to evaluate the physiological implications of the observed phenomena. Aestivation comprises three phases: induction, maintenance and arousal. During the induction phase, animals detect environmental cues and turn them into some sort of internal signals that would instill the necessary changes at the behavioral, structural, physiological and biochemical levels in preparation of aestivation. After entering the maintenance phase, they have to preserve the biological structures and sustain a slow rate of waste production to avoid pollution of the internal environment. Upon the return of favourable environmental conditions, they must arouse from aestivation, excrete the accumulated waste products, and feed for repair and growth. Completion of aestivation occurs only if arousal is successful; if not, the animal would have had apparently succumbed to certain factors during the maintenance phase (Appendix 2). It can therefore be deduced that adaptive changes in nitrogen metabolism, especially protein synthesis and degradation, would vary in different phases of aestivation, although studies in the past focused largely on the maintenance phase.

2.3.3. Preservation of biological structures or conservation of metabolic fuels?

During long-term fasting, animals incapable of aestivation or hibernation enter into a protein catabolic state, mobilizing amino acids as metabolic fuels and releasing ammonia of endogenous origins. However, unlike carbohydrates and lipids, there is no known protein store in animals, and proteins have to be mobilized from biological structures that have specific functions. Skeletal, smooth and cardiac muscles are protein structures with contractile properties but cardiac muscles must be spared from the catabolic process until very critical moments. Although skeletal muscle is the most prominent protein source,

aestivating animals have to preserve muscle structure and strength in preparation of arousal. This has to be achieved in spite of the aestivating animal being in a state of corporal torpor which is associated with skeletal muscle disuse. Muscle disuse can lead to a decrease in protein synthesis and an increase in protein degradation, resulting in muscle atrophy (Childs, 2003). However, a drastic increase in proteolysis, as in the case of fasting alone, does not occur in aestivating animals, as they can effectively preserve muscle structure and strength through suppression of protein degradation and amino acid catabolism. Therefore, suppression of protein degradation during the maintenance phase of aestivation should be regarded primarily as an adaptation to preserve proteinaeous structures and functions (Hudson et al., 2005; Symounds et al., 2007), and conservation of metabolic fuel stores can at best be regarded as a secondary phenomenon.

2.3.4. Modifications of structures/functions or static preservation of structures?

In the past, the occurrence of organic structural modifications in aestivating animals has been largely neglected, but to date, aestivation in African lungfishes is known to be associated with structural and functional modifications in at least the heart and the kidney (Icardo et al., 2008; Ojeda et al., 2008; Amelio et al., 2008). Recently, Icardo et al. (2008) reported that the myocytes in the trabeculae associated with the free ventricular wall of *P. dolloi* showed structural signs of low transcriptional and metabolic activity (heterochromatin, mitochondria of the dense type) while in water. These signs are partially reversed in aestivating fish (euchromatin, mitochondria with a light matrix), and paradoxically, aestivation appears to trigger an increase in transcriptional and synthetic myocardial activities, especially at the level of the ventricular septum (Icardo et al., 2008). In addition, Ojeda et al. (2008) demonstrated structural modifications in all the components of the renal corpuscle of aestivating *P. dolloi*. These changes can be reversed after arousal, indicating that the renal corpuscle is a highly dynamic structure capable of modifying its architecture in

response to different phases of aestivation. Thus, aestivation cannot be regarded as the result of a general depression of metabolism, but it involves the complex interplay between up-regulation and down-regulation of diverse cellular activities (Icardo et al., 2008). Unlike fasting in non-aestivators, aestivation could involve variations in rates of protein degradation and protein synthesis, reconstructing and regenerating cells and tissues during the induction and arousal phases, respectively, through a rapid protein turnover with little production of nitrogenous wastes.

2.3.5. Increased detoxification of ammonia or decreased ammonia production?

Due to the lack of water to facilitate nitrogenous waste excretion, ammonia must be turned into less toxic products for retention. In the past, ammonia detoxification took center stage in nitrogen metabolism in aestivating animals (Wither, 1998; Wright, 2007), but the conversion of ammonia to less toxic products, e.g. glutamine, urea, and uric acid, is energy intensive. More importantly, since aestivating animals undergo long-term fasting, problems associated with toxic ammonia being released from excess amino acids as in fed animals no longer prevail, and there would be a low demand for ammonia detoxification. Furthermore, modification and preservation of biological structures during the induction and maintenance phases of aestivation, respectively, would lead to a low rate of ammonia production which would further ameliorate the demand for ammonia detoxification through energy intensive processes.

2.3.6. Nitrogenous wastes for excretion or nitrogenous products with specific functions?

To date, the intrinsic mechanisms by which cells, tissues and organs are able to adapt and match their function to the environmental cues during aestivation are still enigmatic. Röszer et al. (2004, 2005) reported that nitric oxide (NO) was involved in the neural transmission to intestinal muscles of the snail *Helix lucorum*. During dormancy, enteric

release of NO was blocked and the L-arginine/NO conversion ability of nitric oxide synthase (NOS) was apparently inhibited. Results obtained recently from African lungfish indicate that NO and urea can act as signaling molecules in various phases of aestivation. Amelio et al. (2008) demonstrated that cardiac endothelial NOS (eNOS) expression increased in *P. dolloi* after 6 days of aestivation but decreased in those aestivated for 40 days. Furthermore, both renal localization and expression of eNOS increased with aestivation. They (Amelio et al., 2008) concluded that NO contributed, probably in an autocrine-paracrine fashion, to cardiac and renal readjustments during aestivation. On the other hand, Ip et al. (2005d) reported that increased tissue urea contents could be one of the essential factors in initiating and maintaining aestivation in *P. dolloi*, and there are indications that urea accumulation facilitates rehydration during the arousal phase of aestivation. In addition, Muir et al. (2008) reported that urea depressed the metabolism of living organs *in vitro,* although its effect varied with temperature and seasonal acclimatization. Thus, the conception that urea is accumulated simply as an end-product of ammonia detoxification, pending excretion during subsequent arousal, needs to be re-evaluated.

At present, why aestivators generally prefer to accumulate urea instead of other nitrogenous products during aestivation is debatable. So far, only some phyllomedusid tree frogs are known to coat their body surface with skin secretion and excrete uric acid to minimize water loss during aestivation (Shoemaker et al., 1972; Abe, 1995). Urea accumulation in aestivating animals has been proposed to serve the purpose of reducing evaporative water loss (Campbell, 1973; Storey, 2002), but reports on this phenomenon are controversial. Storey (2002) proposed that a gradual increase in protein catabolism would occur in aestivating animals as the demand for urea synthesis increases, presumably to facilitate retention of tissue water (Storey, 2002). However, urea synthesis is an energy intensive process, utilizing 4 and 5 mol of ATP per mole of urea synthesized in animals possessing carbamoyl phosphate synthetase I (CPS I; e.g. mammals) and CPS III (e.g. snails

and African lungfishes), respectively. An up-regulation of urea synthesis during aestivation would therefore increase energy expenditure and contribute negatively to metabolic depression. More importantly, the mobilization of nitrogen for increased urea synthesis to reduce water loss would contradict the fundamental principles of preservation of biological structures and metabolic fuels during suspended animation. The importance of the preservation of nitrogen during suspended animation is evidenced from hibernating bears, in which urea recycling occurs between animal tissues and the intestinal microbial fauna (Barboza et al., 1997). Urea recycling effectively prevents the build up of urea in the body during hibernation. It minimizes body protein loss and conserves mobility, providing greater flexibility during winter and facilitating rapid resumption of foraging and growth in spring (Barboza et al., 1997). By contrast, urea recycling has not been demonstrated definitively in aestivating animals, indicating that urea accumulated during aestivation could have important functions**.**

2.4. The present study

2.4.1 Excretory nitrogen metabolism in African lungfishes

African lungfishes are obligatory air-breathers. They are ureogenic and possess a full complement of hepatic ornithine-urea cycle enzymes (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989) that comprises CPS III instead of CPS I (Chew et al., 2003b; Loong et al., 2005). However, they are ammonotelic in water, and would turn transiently ureotelic after feeding (Lim et al., 2004; Iftika et al., 2007). African lungfishes (*Protopterus* spp.) can undergo aestivation in mud cocoons during desiccation (Smith, 1930; Janssens, 1964; DeLaney et al., 1974; Fishman et al., 1987), and they can aestivate for as long as three to five years (Smith, 1930), which happens to be the longest aestivation period known among vertebrates. Recently, we have succeeded in inducing African lungfishes to aestivate in completely dried mucus cocoon in plastic boxes in the laboratory (Chew et al., 2004; Ip et al.,

2005f; Loong et al., 2005, 2007, 2008a, b). During the induction phase, the fish hyperventilates and secretes a lot of mucus which turns into a dry mucus cocoon within 6-8 days. Aestivation begins when the fish is completely encased in a cocoon, and there is a complete cessation of feeding and locomotor activities. The fish can continue to aestivate under such conditions for more than a year in the laboratory. The aestivating lungfish can be aroused by the addition of water. Upon arousal, the fish struggles out of the cocoon and swims, albeit sluggishly, to the water surface to gulp air. Feeding begins approximately 10-14 days after arousal, and the fish grows and develops as normal thereafter.

This study focused on excretory nitrogen metabolism in *Protopterus annectens* which our laboratory has secured a constant supply in the past several years. The objectives of this study were:

(1) to determine enzymatically whether *P. annectens* possessed CPS III instead of CPS I,

(2) to evaluate whether *P. annectens* would upregulate urea synthesis during a prolonged induction phase of aestivation (i.e. 6 days of aerial exposure with daily addition of water to prevent total desiccation),

(3) to examine whether the rates of urea synthesis and ammonia production in *P. annectens* would vary between the induction and maintenance phases of aestivation in air,

(4) to elucidate whether 12 or 46 days of aestivation (inclusive of 6 days of induction) in mud would have different effects on excretory nitrogen metabolism in *P. annectens* as compared with aestivation in air,

(5) to determine the effects of aestivation, particularly during the induction and early maintenance phases, in normoxia or hypoxia on energy status, and rates of urea synthesis and ammonia production in *P. annectens*,

(6) to compare and contrast the effects of 6 days of aestivation in normoxia and 6 days of aestivation in hypoxia on up- and down-regulation of gene expressions in the liver of *P. annectens*, using suppression subtractive hybridization (SSH), and

(7) to examine the up-and down-regulation of mRNA expressions of enzymes related to urea synthesis in the liver during the induction, maintenance and arousal phases of aestivation by quantitative RT-PCR (qPCR).

The above-mentioned objectives are organized into 6 individual Chapters in the thesis. Each Chapter is self-sustained with Introduction, Materials and methods, Results, Discussion, and Summary. There is a certain degree of redundancy in the Introduction of these Chapters, but it is unavoidable as the author aimed to organize each Chapter as an independent unit. It is because of that the author made a special effort to end the thesis with a Chapter on "Integration, synthesis and conclusions". The titles of these Chapters are as follow:

Chapter 1: Ornithine-urea cycle and urea synthesis in the African lungfish, *P. annectens*, exposed to terrestrial conditions for 6 days,

Chapter 2: Increased urea synthesis and/or suppressed ammonia production in the African lungfish, *P. annectens*, during aestivation in air or mud,

Chapter 3: Effects of hypoxia on the energy status and nitrogen metabolism of *P. annectens* during aestivation in a mucus cocoon,

Chapter 4: Up- and down-regulation of gene expressions in the liver of *P. annectens* after 6 days of aestivation in normoxia or hypoxia,

Chapter 5: mRNA expression of genes related to urea synthesis in the liver *P. annectens* during the induction, maintenance (6 month) and arousal phases of aestivation, and

Chapter 6: Overall integration, synthesis and conclusions.

It was hoped that the present study will have a substantial contribution to the understanding of aestivation, specifically in relation to excretory nitrogen metabolism, in African lungfishes, and shed light on answers to some of the enigmatic issues mentioned above.

NOTE:

Chapter 1 has been published as: Loong, A. M., Hiong, K. C., Lee, S. M. L., Wong, W. P., Chew, S. F., and Ip, Y. K. (2005). Ornithine-urea cycle and urea synthesis in African lungfishes, *Protopterus aethiopicus* and *Protopterus annectens*, exposed to terrestrial conditions for 6 days. J. Exp. Zool. 303A, 354-365.

Chapter 2 has been published as: Loong, A. M., Ang, S. F., Wong, W. P., Pörtner, H. O., Bock, C., Wittig, R., Bridges, C. R., Chew, S. F., and Ip, Y. K. (2008a). Effects of hypoxia on the energy status and nitrogen metabolism of African lungfish during aestivation in a mucus cocoon. J. Comp. Physiol. 178B, 853-865.

Chapter 3 has been published as: Loong, A. M., Pang, C. Y. M., Hiong, K. C., Wong, W. P., Chew, S. F., and Ip, Y. K. (2008b). Increased urea synthesis and/or suppressed ammonia production in the African lungfish, *Protopterus annectens*: aestivation in air versus aestivation in mud. J. Comp. Physiol. 178B, 351-363.

3. Literature review

3.1. Production and excretion of ammonia in fish

3.1.1. Excess dietary protein and gluconeogenesis

Amino acids have numerous functions; they are the building blocks of proteins that are needed for survival, growth and development. Dietary protein is a major source of amino acids in animals. Under normal circumstances, most animals take in amino acids in excess of what is needed to sustain growth and protein turnover. Unlike carbohydrates and lipids, which can be stored as glycogen and triglycerides, respectively, amino acids are not stored to any great extent and animals are not known to possess protein stores solely for the purpose of energy metabolism (Campbell, 1991). Therefore, excess amino acids from diets are preferentially degraded, and their carbon skeletons can be channeled directly into the tricarboxylic acid cycle or converted to glucose through gluconeogenesis (Campbell, 1991). Several amino acids, including alanine, are converted to glucose by fish hepatocyes (French et al., 1981) and this process is regulated hormonally in much the same way as it is in mammals. Approximately 40-60% of the nitrogen intake from food is excreted within 24 h (Lim et al., 2004; Ip et al., 2004c). In addition to diet, muscle proteins can act as a source of amino acids, which are catabolized for the production of ATP or carbohydrates, in fasting fishes (Houlihan et al., 1995). During exercise or hypoxia, ammonia can also be produced through the deamination of AMP in the skeletal muscle. In vertebrates, the liver acts as the "glucostat" where amino acid catabolism and gluconeogenesis take place (Campbell, 1991). Amino acids reaching the liver via the hepatic portal system from the intestine or via the systemic circulation from the extra-hepatic tissues serve as major gluconeogenic substrates. Glucose can then be supplied to other tissues or stored as glycogen.

3.1.2. Ammonia production and related excretory products

The first step in amino acid catabolism involves the removal of the α-amino nitrogen as ammonia. For some amino acids, deamination involves specific deaminases, but many amino acids are deaminated through transdeamination (Campbell, 1973, 1991). Transdeamination of amino acids usually occurs in the liver and requires an initial transamination of the amino acid with α-ketoglutarate in the cytosol to form glutamate, which then enters the mitochondria and is oxidatively deaminated by glutamate dehydrogenase (GDH). GDH is therefore crucial to the regulation of amino acid catabolism, and hence ammonia production. It also plays an important role in integrating nitrogen and carbohydrate metabolism (Appendix 1). Amino acid catabolism releases ammonia which, because of its toxicity, must be disposed of or detoxified.

Much of the ammonia produced in fish comes from the α-amino group of amino acids that are catabolized. The rate of alanine deamination by catfish hepatocytes can account for 50% of the total ammonia excreted by live fish and the rest with glutamine, 85% (Campbell et al., 1983). In addition, the rate of glutamate deamination by intact catfish liver mitochondria can account for 160% of the rate of ammonia excretion (Campbell et al., 1983). For goldfish, the liver is responsible for 50-70% (Van den Thillart and van Raaji, 1995) of ammonia production. So, liver is a main site of ammonia formation in fish. Ammonia is produced either directly in the cytosol of hepatocytes by specific deaminases (histidase, asparaginase, serine dehydratase and threonine dehydratase; Youngson et al., 1982) or via the combined actions (transdeamination) of cytosolic aminotransferases and mitochondrial glutamate dehydrogenase (GDH) (French et al., 1981; Walton and Cowey, 1982). Transdeamination is the primary mechanism for catabolism of amino acids in fish liver. GDH is localized exclusively in the matrix of fish liver mitochondria, so it is within this compartment that ammonia is released through the route of transdeamination. Glutaminase, which release NH_3 from the amide-function of glutamine is also present in the mitochondrial matrix of some fish species. Thus, ammonia released in the matrix has to exit the

mitochondria, a process which may be deleterious to oxidative phosphorylation. Furthermore, ammonia is toxic for many other reasons and therefore it has to be excreted or converted into less toxic compounds for transient storage before excretion.

Ammonotelic species prevent the buildup of ammonia in their bodies by efficiently excreting ammonia, usually in an aquatic medium. Some animals can facilitate NH_3 excretion by increasing H^+ excretion (Chew et al., 2003a; Ip et al., 2004b; Tay et al., 2006; Wood et al., 2005a), while a few animals are known to be capable of actively excreting ammonia against an unfavourable NH_4^+ gradient (Randall et al., 1999; Ip et al., 2004b, d; Tay et al., 2006; Chew et al., 2007). In terrestrial species that are ureotelic and/or uricotelic, cooperativity between enzymes in the mitochondrial and cytosolic compartments leads to the formation of urea and uric acid, respectively. For ureotelic and uricotelic species, the transient accumulation of end-products in their body fluids posts a much lesser problem than ammonia since urea and uric acid are relatively less toxic. Ammonia can be detoxified to urea through the ornithine-urea cycle in certain land snails, African and South American lungfishes, coelacanths, amphibians, chelonid and rhynchocephalid reptiles and mammals. Circumstantial physiological evidence suggest that active urea transport systems may exist in mammals (Sands, 2003), amphibians (Schmidt-Nielsen and Shrauger, 1963; Katz et al., 1981; Rapoport et al., 1988; Lacoste et al., 1991), elasmobranchs (Schmidt-Nelsen et al., 1972; Morgan et al., 2003; Part et al., 1998; Fines et al., 2001) and teleosts (McDonald and Wood, 1998; McDonald et al., 2000, 2002, 2003). However the molecular basis for active urea transport is unknown although urea transporters (UT-A) that enable the facilitated diffusion of urea have been identified in the mammalian kidney (You et al., 1993; Smith et al., 1995), amphibian bladder (Couriaud et al., 1999; Konno et al., 2006), elasmobranch kidney (Smith and Wright, 1999; Morgan et al., 2003; Hyodo et al., 2004; Birukawa et al., 2008) and teleost gills (Walsh et al., 2000, 2001a, 2001b). In teleosts, the expression of urea transporters was thought to be restricted to the gill until a cDNA ortholog, known as UT-C was identified in

eel kidney (Mistry et al., 2005). Excretion of urea requires at least a limited supply of water. Perhaps, because of that, the ornithine-urea cycle (OUC) became dysfunctional in the reptilian line (Squamata and Crocodilia) leading to the birds, and these animals detoxify ammonia to uric acid instead of urea (Campbell, 1973, 1995). Uric acid is highly insoluble in water and can therefore be excreted in a semi-solid state.

3.1.3. Passage of NH_3 and NH_4^+ through biomembranes

In aqueous solution, total ammonia has two components —the gas NH_3 and the cation NH_4^+. The equilibrium reaction can be written as $NH_3 + H_3O^+ \Leftrightarrow NH_4^+ + H_2O$, and the pK of this NH_3/NH_4^+ reaction is around 9.0 to 9.5. The properties of ammonia that determine its transport across biological membranes are that NH_3 react avidly with water and is moderately soluble in lipid, and that NH_4^+ has some ionic properties similar to those of K^+ and can therefore compete with K^+ on membrane ion channels and transporters (Marcaggi and Coles, 2001). NH_3 has a high solubility in water and is weakly soluble in lipids. In this respect, it contrasts strongly with lipophilic molecules such as CO_2; and, so, the permeability to NH_3 of nearly all cell membranes is much less than their permeability to CO_2 or O_2, other gases of physiological importance. Nevertheless, biomembranes are so thin that NH_3 can diffuse quite rapidly through nearly all of them, although the NH_3 permeability varies greatly and can be very low in some cases (Marcaggi and Coles, 2001). Because phospholipids of biological membranes are not very permeable to NH_4^+, therefore, in most cases, ammonia crosses membranes as NH_3. However, a small amount of NH_4^+ can permeate biomembranes through K^+ channels (Thomas, 1984), and so, in some cases, exogenous ammonia would result in intracellular NH_3 cycling and a decrease in the intracellular pH (Marcaggi and Coles, 2001). According to Choe et al. (2000), most of the values for P_{NH4}/P_K through K^+ channels range between 0.1 and 0.3. However, some K^+ channels apparently has high specificity for K^+, for

example, those of the starfish egg, which have a P_{NH4}/P_K value of 0.03, and the glial cells of bee retina (see Marcaggi and Coles, 2001 for a review).

There is now direct evidence that NH_3 can traverse the membrane through water channel proteins or aquaporins (AQP1; Nakhoul et al., 2001). Besides aquaporins and K^+ channels, the Rhesus glucoproteins (RhAG, RhBG, RhCG) belonging to the ammonia transporter/methylammonium permease/Rhesus glucoprotein (AMT/RH) superfamily are known to be involved in ammonia transport across biomembranes (Marini et al., 1997). Human RhBG and RhCG are expressed in diverse tissues, while RhAG is limited to red blood cells (Huang and Liu, 2001; Liu et al., 2000). The mechanism of ammonia transport by Rh glycoproteins is still unclear (Planelles, 2007). At present, there are three hypotheses: (1) an electrogenic NH_4^+ movement (Nakhoul et al., 2005), (2) an electroneutral NH_4^+/H^+ mediated exchange (Ludewig, 2004), and (3) a direct NH_3 transport associated with NH_4^+ transport (Bakouh et al., 2004).

Membranes of several cell types facing the gastric and urinary tracts have been found to have relatively low permeability to NH_3. Kikeri et al. (1989) found that when ammonium was applied in the lumen of the medullary ascending limb of Henle of the mouse, the initial intracellular pH (pH_i) change was in the acid direction. This acid change could be blocked pharmacologically by the application of furosemide. NH_4^+ then had no effect on pH_i. Therefore, Kikeri et al. (1989) concluded that the membranes were relatively impermeable to NH_3. Despite a subsequent suggestion by Good (1994) that there could be rapid efflux of NH_3 through the basolateral membranes in these experiments, later works have indeed substantiated the existence of plasma membranes in the urinary tract of the rabbit (Yip and Kurtz, 1995) and other animal cell membranes with low NH_3 permeability. A particular elegant demonstration of such a low NH_3 permeability is that of Singh et al. (1995) on the luminal (apical) surface of colonic crypt cells of the rabbit. The apical membranes of bladder cells of the rabbit, which also have a low NH_3 permeability, have an unusual composition

with 70-90% of the membrane area being occupied by paracrystalline arrays of proteins call uroplakins (Chang et al., 1994). It therefore seems likely that relatively high NH_3 permeability is a normal property of cell membranes that is only reduced when the phospholipid composition is altered and/or when lipids are replaced by proteins (Marcaggi and Coles, 2001).

3.1.4. Excretion of ammonia in ammonotelic fishes

The gills are the primary site of ammonia excretion in fish (Wilkie, 1997, 2002), because they have a large surface area, perfusion by 100% of cardiac output, large ventilation rates, small diffusion distances, and contact with a voluminous mucosal medium (Evans et al., 2005). Although gill tissues exert an extremely high metabolic rate, accounting for almost 10% of the entire oxygen demand of teleosts for osmoregulatory purposes, the overall metabolic expenditures for the release of ammonia appear to be minimal (Evans et al., 2005). Most fishes, with a few exceptions, are ammonotelic. The majority of ammonia is excreted across the branchial epithelium as NH_3, down a favourable blood-to-water diffusion gradient (Wilkie, 1997, 2002; Evans et al., 2005), and there is probably minimal NH_4^+ diffusion in freshwater fishes. In freshwater fishes, excreted NH_3 can be trapped via H^+ secretion or CO_2 excretion into the unstirred layer of water on the apical surface of the gills (Avella and Bornancin, 1989). H^+ secretion can be achieved through an apical vacuolar type proton ATPase (V-ATPase; see Lin and Randall, 1995 for a review), although there is an apparent lack of Na^+/H^+ (NH_4^+) exchange via Na^+/H^+ exchangers (NHE) in gills of freshwater fishes (Evans et al., 2005). In general, the branchial V-ATPase is preferentially expressed in the gills of freshwater, and not marine, fishes, and it has been linked to the uptake of Na^+ and Cl^- as well as acid-base regulation (Evans et al., 2005).

Rh proteins have been shown to be expressed in fish (Kitano and Saitou, 2000), and they apparently also participated in ammonia excretion. They are present in the gills of *Takifugu rubrips* (Nakada et al., 2007b) and *Onchorhynchus mykiss* (Nawata et al., 2007), and the yolk sac, gills, kidney and skin of *Danio rerio* (Nakada et al., 2007a; Hung et al., 2007; Shih et al., 2008). There is evidence which suggests a cooperation between Rh proteins and V-ATPase in ammonia excretion in fish (Nawata et al., 2007; Shih et al., 2008), and confirms the important role of V-ATPase in boundary layer acidification that would facilitate ammonia excretion.

For marine fishes, despite the presence of sodium-hydrogen exchanger (NHE), which facilitates Na^+ absorption, little to no ammonia excretion is via Na^+/NH_4^+ exchange (Wilkie, 2002), because of the presence of favourable NH_3 and NH_4^+ diffusion gradients. Unlike freshwater fishes, seawater fishes have shallow tight junctions between mitochondria-rich cells, which increase cation permeability for Na^+ secretion. Therefore, a significant portion of ammonia can be excreted through NH_4^+ diffusion through the paracellular route in seawater fishes (Goldstein et al., 1982).

The branchial Na^+/K^+-ATPase is important in iono-regulation providing the driving force for secondary active Cl^- excretion in marine fishes and Na^+ uptake in freshwater fishes (Evans et al., 2005). Due to the similarities in hydration radius and electrical charge between K^+ and NH_4^+, Na^+/K^+-ATPase has also been implicated in ammonia excretion in fish. In the giant mudskipper, Na^+/K^+-ATPase has a role in active ammonia excretion through the gills (Randall et al., 1999).

Aquaporins (AQP3) have been reported in fish gills (Cutler and Cramb, 2002), but only in the basolateral membrane and intracellular vesicles (Lignot et al., 2002). In a recent review, Wilkie (2002) has given the AQP an apical localization, which may be suitable in aiding transepithelial NH_3 fluxes but would be disastrous for water fluxes because of the presence of large osmotic gradients across the gills of both marine and freshwater fishes. In

light of the barrier function of apical versus the basolateral membrane, it is unlikely that branchial AQPs have a significant role in transepithelial NH_3 fluxes.

3.2. Impediment of ammonia excretion and mechanisms of ammonia toxicity in fish

3.2.1. Environmental conditions that impede ammonia excretion or lead to an influx of ammonia

While excretion of ammonia is not a problem at low environmental pH, it is a major problem for fish exposed to high pH. This is because at high pH, the gradient for NH_3 diffusion is reduced and this may lead to a build-up of ammonia inside the fish (Wilkie and Wood, 1995). Death can occur when the rise in plasma ammonia level is too rapid and/or ammonia toxic levels are reached (Wilkie et al., 1993).

Air-breathing is one of several adaptive responses utilized by fishes dwelling in habitats where O_2 supplies may be severely depleted (Graham, 1997). While most air-breathing fishes remain aquatic, some evolved to emerge from water, make excursion onto land, or even burrow into mud when the external media dry up. When a fish is out of water, it is confronted with problems of endogenous ammonia excretion because there is a lack of water to flush the branchial and cutaneous surfaces. So, fishes must have adaptations to ameliorate ammonia toxicity during long term emersion (see Ip et al., 2004a and Chew et al., 2006 for reviews).

Some air-breathing fishes can be trapped in puddles of water occasionally, or in crevices for many days; continual excretion of endogenous ammonia into a small volume of external media can lead to high external ammonia concentrations. Furthermore, water evaporation at the high temperatures of the tropics can concentrate environmental ammonia to high levels. In addition, fishes can be exposed to high concentrations of environmental ammonia under several conditions. Some tropical fishes may have unique behaviors; for

example, mudskippers build burrows in the mud in estuaries and stay therein during high tides. During the breeding season, the male fish stay inside the burrow for 1-2 months to take care of the developing embryos and fry. Since the burrow water is not well flushed, the ammonia concentrations can be high, and mudskippers have to deal with the toxicity of environmental ammonia in the burrow. Some fishes live in rice fields, where agricultural fertilization can lead to high concentrations of environmental ammonia. In the presence of high concentrations of environmental ammonia, fishes are confronted simultaneously with retention of endogenous ammonia and uptake of exogenous ammonia, and they have special adaptations to deal with ammonia toxicity.

3.2.2. Deleterious effects of endogenous ammonia

Ammonia is toxic for many reasons (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip et al., 2001b; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002). At the molecular level, NH_4^+ can substitute for K^+ in Na^+, K^+-ATPase and in $Na^+/K^+/2Cl^-$ co-transport (see Wilkie, 1997, 2002 for reviews; Person-Le Ruyet et al., 1998), and for H^+ in Na^+/ H^+ exchanger (Randall et al., 1999). In neurons, NH_4^+ can substitute for K^+ and permeate through K^+ background channels, affecting the membrane potential (Binstock and Lecar, 1969). Ammonia can interfere with energy metabolism through inhibiting certain glycolytic enzymes and impairment of the tricarboxylic acid cycle (Campbell, 1973; Arillo et al., 1981).

In vertebrates, ammonia toxicity normally manifests as encephalopathy at the organismal level; the animal enters into a coma and succumbs to the deleterious effects of ammonia. Two distinct mechanisms underlying ammonia toxicity in the central nervous system of mammals have been identified. The primary and rapid event involves the over-activation of N-methyl-D-aspartic acid (NMDA) receptor in neurons (Fan and Szerb, 1993; Hermenegildo et al., 2000), and probably also in astrocytes (Schliess et al., 2002; 2004), due

to increases in the concentration of extracellular glutamate (Michalak et al., 1996) resulting from an inhibition of glutamate uptake (Oppong et al., 1995) or an increase in glutamate release from neurons (Ross, 2002). The overactivation of NMDA receptor leads to an increased overproduction of nitric oxide (NO) and toxic reactive oxygen (ROS) and/or nitrogen (RNOS) species (Murthy et al., 2001; Hilgier et al., 2003; Kosenko et al., 2003; Haussinger et al., 2005; Swamy et al., 2005), which in turn leads to extensive destruction of proteins (Kosenko et al., 1999, 2000) and oxidation of RNA (Görg et al., 2008) in neurons and astrocytes. The early increase in accumulation of cGMP is a marker of this condition (Hermenegildo et al., 2000; Hilgier et al., 2003, 2005), and cGMP may also contribute to the neurophysiologic manifestation of encephalopathy (Albrecht et al., 2007). It has been demonstrated that intracerebral administration of NH_4^+ via a microdialysis probe causes an instant activation of the NMDA/NO/cGMP pathway (Hilgier et al., 2003, 2004; 2005). The second mechanism of ammonia neurotoxicity is attributable to the ammonia-induced increases in glutamine synthesis and accumulation, resulting in astrocytic swelling and cerebral edema (Brusilow, 2002; Tofteng et al., 2006; Albrecht and Norenberg, 2006). At the cellular level, excess glutamine can mediate mitochondrial damage and mitochondrial generation of deleterious ROS in astrocytes (Rama et al., 2003; Jayakumar et al., 2004). Inhibition of glutamine transport into mitochondria protects astrocytes from ammonia toxicity (Pichili et al., 2007). In addition, *in vivo* inhibition of glutamine synthetase prevents not only ammonia-induced astrocyte swelling (Tanigami et al., 2005) but also cerebral NO production (Master et al., 1999) and protein tyrosine nitration (Schliess et al., 2006). Thus production of NO and ROS appears to be the common mechanism for both the NMDA receptor- and the glutamine-mediated pathways of the ammonia neurotoxicity. Recently, Hilgier et al. (2008) demonstrated that there could be mutual interaction between these two pathways, since glutamine, at physiological concentrations, can ameliorate excessive activation of the NO-cGMP pathway by neurotoxic concentrations of ammonia.

Unlike mammals, some tropical air-breathing fishes can tolerate high levels of ammonia (see Ip et al., 2001b, 2004a, e, and Chew et al., 2006 for reviews). Some of these fishes can synthesize and accumulate high levels of glutamine in their brains and extra-cranial tissues (Peng et al., 1998; Anderson et al., 2002; Tsui et al., 2002; Tay et al., 2003; Ip et al., 2001a, e; Wee et al., 2007). Thus, the mechanisms of ammonia toxicity in the brains of fish species with high ammonia tolerance are likely to be different from those in mammalian brains (Veauvy et al., 2005; Ip et al., 2005a; Wee et al., 2007; Tng et al., 2009).

3.2.3. Deleterious effects of environmental ammonia

Environmental ammonia has deleterious effects on branchial ion transport not associated with the accumulation of endogenous ammonia. These effects are not applicable to fish simply exposed to terrestrial conditions or to fish injected/infused with exogenous ammonia. Acute exposure to environmental ammonia results in inhibition of Na^+ influx in the temperate rainbow trout *Oncorhynchus mykiss* (Avella and Bornancin, 1989) and the goldfish *Carassius auratus* (Maetz, 1973). In *C. auratus*, the deleterious effect is specific to Na^+ uptake and not general to the epithelium or all ion uptake mechanisms. In contrast, no deleterious effect of ammonia exposure (up to 28.2 μmol l^{-1} NH_3-N or 5.2 mmol l^{-1} total ammonia) is seen on Na^+ uptake in juvenile rainbow trout, but Na^+ efflux is stimulated by ammonia levels greater than 6.4 μmol l^{-1} NH_3-N (1.2 mmol l^{-1} total ammonia) (Twitchen and Eddy, 1994). This increase in efflux is likely through an increased Na^+ permeability of the gills (Gonzalez and McDonald, 1994), mediated through a modulation of the paracellular pathway (Madara, 1998). In addition, exposure to environmental ammonia predisposes the gills to histopathological changes that may disrupt ion transport (Daoust and Ferguson, 1984). Disruption of epithelial integrity has adverse consequences for ion transport and other cellular processes, and the proliferation of branchial mucous cells induced by environmental ammonia increases diffusion distances across the gill (Ferguson et al., 1992).

3.3. Defense against ammonia toxicity in fish

3.3.1. Active transport of NH_4^+

Theoretically, the most effective way to defend against ammonia toxicity in fish in alkaline water, during emersion or when exposed to ammonia-loading conditions is active transport of NH_4^+ (as have been suggested for the inner mitochondrial membrane of ammonotelic fishes; Campbell, 1997), because it facilitates the maintenance of low internal ammonia levels and protects the brain from ammonia toxicity. Indeed, some air-breathing tropical fishes with modified gill structures or accessory-breathing-organs are capable of doing so (Chew et al., 2003a; Randall et al., 1999; Ip et al., 2004d, f).

The gills of the giant mudskipper *P. schlosseri* are specially adapted for terrestrial survival (Ip et al. 1993; Kok et al. 1998) because of its specialized gill morphology and morphometry (Low et al. 1988, 1990; Wilson et al. 1999, 2000). There are intrafilamentous interlamellar fusions in its gills, which form numerous fenestrae on the surface of the gill filament. When 8 μmol g^{-1} ammonium acetate was injected intraperitoneally into *P. schlosseri* followed with 24 h of aerial exposure, the cumulative ammonia excreted is significantly greater than the saline-injected control (Chew et al., 2007). At hour 2, the ammonia excretion rate in the experimental fish is transiently greater than that in fish kept in water. By hour 6, a large portion (33%) of the injected ammonia was excreted by the experimental fish through the head region, probably through the gills (Chew et al., 2007). Since ammonia could be excreted only into the small amount of water trapped in the fenestrae of the fused secondary lamellae in the gills of *P. schlosseri* during emersion, it is logical to deduce that ammonia concentration build up quickly therein and reached high levels. So, *P. schlosseri* can effectively excrete a high load of ammonia on land, which indirectly supports the proposition that active NH_4^+ excretion contributes in part to its high terrestrial affinity and high tolerance of aerial exposure.

In addition, *P. schlosseri* can maintain an unchanged ammonia excretion rate when exposed to alkaline water (Chew et al., 2003a). It can also tolerate very high concentrations of environmental ammonia (>100 mmol l^{-1} NH_4Cl; Ip et al., 1993; Peng et al., 1998). When exposed to environmental ammonia, *P. schlosseri* is able to maintain low concentrations of ammonia in its plasma (Peng et al., 1998; Randall et al., 1999) as a result of its ability to actively excrete ammonia against large inward NH_3 and NH_4^+ gradients (up to at least 30 mmol l^{-1} NH_4Cl pH 7.2; Randall et al., 1999). The mechanisms apparently involve Na^+, K^+ (NH_4^+)-ATPase at the basalemma and Na^+/H^+ (NH_4^+) exchangers at the apical membrane of the branchial epithelia. NH_4^+ substitutes for K^+ in a number of ion transport proteins owing to similarity in their hydration radii. Indeed, active ammonia excretion in *P. schlosseri* exposed to environmental ammonia is sensitive to ouabain, an inhibitor of Na^+, K^+-ATPase, and NH_4^+ can replace K^+ as a substrate for ATPase activity *in vitro* (Randall et al., 1999). Moreover, the addition of amiloride, an inhibitor of NHE, to the external medium decreases the rate of ammonia excretion in *P. schlosseri* (Randall et al., 1999). NHE2- and NHE3-like proteins have been shown to be present in the apical crypts of branchial mitochondria-rich cells (Wilson et al., 2000). Since plasma Na^+ level increases in specimens exposed to high concentrations of environmental ammonia (Randall et al., 1999), NH_4^+ must be actively transported across the branchial epithelium in exchange with Na^+. The possibility of NH_3 trapping in the active excretion of ammonia can be eliminated because the addition of buffer to the medium, which removes the acid boundary layer, has no effect on the rate of ammonia excretion (Wilson et al., 2000; Ip et al., 2004d). Also the addition of bafilomycin A1, a H^+-ATPase inhibitor, significantly decreases the net acid flux but has no effect on the rate of ammonia excretion (Ip et al., 2004d).

In the silver shiner, branchial Na^+/K^+-ATPase activity increases with exposure to ammonia (Alam and Frankel, 2006). However, in the rainbow trout, branchial Na^+/K^+-ATPase activity and mRNA expression are not modified by ammonia exposure (Nawata et

al., 2007; Salama et al., 1999). Moreover, in the juvenile European eel (*Anguilla anguilla*), there is actually a negative correlation between Na^+/K^+-ATPase activity and environmental ammonia levels (Moreira-Silva et al., 2009). Hence, the role of branchial Na^+/K^+-ATPase in fish ammonia tolerance is far from clear.

3.3.2. Lowering of environmental pH

For fishes living in stagnant water of a finite volume (puddle, tidepool or water-filled burrow), it is possible for the acidifying effects of their excretions of H^+ and CO_2 to have a significant impact on environmental pH. This lowering of environmental pH has advantages for dealing with elevations in environmental ammonia levels by reducing the concentration of NH_3, the more permeant species of ammonia, constituting "environmental ammonia detoxification" (Chew et al., 2003a).

Mudskippers build burrows on the mud flat, and lay eggs therein during the breeding season. In one study, the pH of the water in a canal that supplied water to a mud flat was 7.84, yet the pH of the water sampled from burrows of the giant mudskipper *P. schlosseri* was close to 7.0 (Ip et al. 2004d). This indicates that *P. schlosseri* modifies the pH of the burrow water, confirming laboratory observations made by Chew et al. (2003a). Lowering the pH of the burrow water from 8.3 (pH of full strength sea water) to 7.0 increases the ratio of NH_4^+ to NH_3, which lowers the toxicity of NH_3 to the fish and embryos in the burrows. When *P. schlosseri* is kept in 10 volumes (w/v) of 50% seawater with 2 mmol l^{-1} of Tris-HCl at pH 8.5 or 9.0, the decrease in pH is large and rapid (Chew et al., 2003a; Ip et al., 2004d). Thus, large quantities of acid must be excreted to manipulate the external pH of alkaline waters. More importantly, *P. schlosseri* is capable of increasing the rate of net acid excretion in response to the presence of 20 or 30 mmol l^{-1} NH_4Cl in the water at pH 7.0 or 8.0 (Ip et al., 2004d). This represents direct evidence linking net acid excretion with defence against environmental ammonia toxicity in *P. schlosseri*.

As a result of active excretion of NH_4^+ into a finite volume of water in the burrow, the ambient ammonia concentration increases. Therefore, it is imperative for the excreted NH_4^+ not to dissociate into NH_3 and H^+, because NH_3 would diffuse back into the body down an inwardly directed ΔP_{NH3}. Because acid excretion is responsive to environmental ammonia, there is a continuous excretion of acid even at neutral pH. This would maintain a low pH (high concentrations of H^+) in the boundary water layer of the branchial epithelia, preventing the excreted NH_4^+ from dissociating into NH_3 and H^+, and avoiding back flux of NH_3. In essence, this is the process of "NH_4^+ trapping". Indeed, both active NH_4^+ excretion and H^+ excretion take place in the head region of *P. schlosseri*, where the gills and the opercular membranes are located (Ip et al., 2004d). It is essential for these two mechanisms to be located together, because the branchial and opercular surfaces have the important functions of allowing passage of gases and other ions. Excretion of acid to trap the actively excreted NH_4^+ is likely to be more effective than modifying the fluidity of these surfaces to change the permeability of NH_3, which would also affect the permeability of other gaseous molecules.

Wood et al. (2005a) reported that the slender African lungfish *P. dolloi* exposed to 30 $mmol \cdot l^{-1}$ NH_4Cl in a closed system without aeration was able to greatly acidify the external water, a volume 25-fold greater than its own volume. The extent of this acidification increased with time. After several days, the external pH fell from about 7.0 to below 5.0 over a 24-h period, thereby markedly reducing the concentration of NH_3 (the form that diffuses across biological membranes). CO_2 excretion is partially responsible for this acidification, because vigorous water aeration reduces but does not eliminate the acidification, and urea-N excretion increases moderately. However, a substantial excretion of titratable acid (non-CO_2 acidity) also occurred. Wood et al. (2005a) therefore concluded that environmental acidification could be a less costly strategy for avoiding ammonia toxicity than detoxifying ammonia through increased urea production.

3.3.3. Low NH_3 permeability of cutaneous surfaces

The branchial epithelial surface of aquatic teleosts has a higher permeability to NH_3 due to its major function in gaseous exchange. Since air-breathing fishes depend largely on the accessory breathing organ for respiration, their gills are usually degenerate (Graham, 1997). Moreover, they usually hold air in their buccal cavities during immersion, which means their gills would not be exposed to the external medium, and NH_3 excretion through the gills would be relatively ineffective. However, many tropical air-breathing fishes substitute branchial respiration with cutaneous respiration by having highly vascularized skins. Therefore, a substantial portion of ammonia excretion takes place through the cutaneous surface of air-breathing fishes (Graham, 1997).

For fishes which are exposed frequently to environmental ammonia, it would be essential for them to reduce the permeability of their skins to NH_3 despite the cell membranes being permeable to gaseous molecules like O_2 and CO_2, even though the permeability of NH_3 is less than those of O_2 and CO_2 (Marcaggi and Coles, 2001). NH_3 permeates the membrane by solvation and diffusion in the lipid bilayer. The lipid-water partition coefficient for ammonia is low (Evans and Cameron, 1986), suggesting that membrane permeability to ammonia is generally low. In certain biological situations, however, membrane permeability to ammonia is further reduced (Kikeri et al., 1989), and several mechanisms for reducing the permeability of membranes to ammonia are apparent from model studies. In particular, the cholesterol and phospholipid fatty acid contents of artificial membranes have been shown to affect the permeability of artificial membranes to ammonia (Lande et al., 1995). When a fish is confronted with ammonia-loading conditions, a reduction in NH_3 permeability of the skin would help reduce the influx of NH_3. In the giant mudskipper, this is an important adaptation which complements active NH_4^+ excretion through its gills (Randall et al., 1999; Ip et al., 2004d), because it would prevent a back diffusion of NH_3 through the cutaneous surfaces after the build up of high ammonia concentrations in the external medium (Ip et al., 2004d).

Indeed, the flux of NH_3 through the skin of *P. schlosseri* in an Ussing apparatus at 25°C down a 10-fold NH_4Cl gradient between two media with 1 unit pH difference (pH 8 → pH 7) is only 0.009 μmol min^{-1} cm^{-2} (Ip et al., 2004d). The low NH_3 permeability in the skin of *P. schlosseri* is likely due to its low membrane fluidity (Lande et al., 1995). The phosphatidylcholine:phosphatidylethanolamine ratio, an indication of membrane fluidity (Hazel and Williams, 1990), from the skin of *P. schlosseri* is 3.4, much higher than those of tissues of other fish species (Hazel and Landrey, 1988). Furthermore, the skin of *P. schlosseri* has very high cholesterol content (4.5 μmol g^{-1}), which lowers the fluidity of biomembranes. The cholesterol content in the skin of *P. schlosseri* increased significantly to 5.5 μmol g^{-1} after 6 days of ammonia exposure (Ip et al., 2004d), suggesting a role for cholesterol as a defence mechanism against environmental ammonia toxicity.

African lungfishes which can tolerate at least 100 mmol l^{-1} NH_4Cl may also have low permeability to NH_3 in the skin. Chew et al. (2005b) estimated the flux of NH_3 through the skin of *P. dolloi* to be only 0.003 μmol min^{-1} cm^{-2}, which is even lower than that of the giant mudskipper *P. schlosseri*. It is probably because of this that *P. dolloi* could afford to detoxify ammonia to urea during exposure to high concentrations of environmental ammonia (Chew et al., 2005b) despite urea synthesis being energetically expensive.

3.3.4. Volatilization of NH_3

Because ammonia can exist as NH_3 gas, it is logical certain fish may be able to excrete NH_3 directly into the atmosphere. In teleosts, ammonia volatilization was first reported in the temperate intertidal blenny (*Blennius pholis*), but it only accounted for 8% of the total ammonia excreted during emersion (Davenport and Sayer, 1986). However, the ammonotelic tropical fishes *Alticus kirki* (the leaping blenny) (Rozemeijer and Plaut, 1993), *Rivulus marmoratus* (the mangrove killifish) (Frick and Wright, 2002) and *Misgurnus anguillicaudatus* (the oriental weatherloach) (Tsui et al., 2002) are capable of volatilizing

significant amounts of ammonia during aerial exposure. High temperatures and humidity increase the likelihood of the ammonia excreted into the film of water covering the body surface of tropical fishes being volatilized.

In the case of *M. anguillicaudatus*, ammonia built up to very high levels in the muscle (14.8 μmol g^{-1}), liver (15.2 μmol g^{-1}) and plasma (5.09 μmol ml^{-1}) after 48 h of emersion (Chew et al., 2001). Building up of internal ammonia may be an essential prerequisite for volatilization to occur. The blood pH simultaneously becomes more alkaline; this would lead to a high level of NH_3 in the blood. It is possible that a much higher ΔP_{NH3} gradient is required to facilitate the efflux of ammonia through non-branchial epithelial surfaces. In *M. anguillicaudatus*, there appears to be at least two sites of NH_3 volatilization, i.e. the skin and the digestive tract, under terrestrial conditions. During emersion, the surface of the skin becomes significantly more alkaline (Tsui et al., 2002). Moreover, the pH of the water underneath the fish is significantly higher than that of the submerged control. Since the fraction of NH_3 present is strongly influenced by the pH of the medium, the skin of *M. anguillicaudatus* can be a site of NH_3 volatilization. Simultaneously, the pH of the mucosal surface of the anterior portion of the digestive tract becomes significantly more alkaline in *M. anguillicaudatus* exposed to terrestrial conditions (Tsui et al., 2002). Although the skin and the digestive tract may both be involved in NH_3 volatilization when the fish is moving on land, excretion of ammonia through the skin would be ineffective when the fish burrows into the mud. In the mud, maintaining the skin surface or the surrounding film of water at pH 8 would aggravate the situation, because of the reversed ΔP_{NH3} gradient. When the fish is surrounded by mud, the digestive tract (either through the mouth or the cloaca) becomes the only avenue for NH_3 volatilization to occur.

3.3.5. Detoxification of ammonia to glutamine

Glutamine formation plays a role in detoxifying exogenous and endogenous ammonia in fish, especially in the brain, during exposure to environmental ammonia (Arillo et al., 1981, Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng et al., 1998) or after feeding (Wicks and Randall, 2002a, b; Lim et al., 2004). Glutamine is produced from glutamate and NH_4^+, the reaction catalyzed by glutamine synthetase (GS). Glutamate may in turn be produced from α-ketoglutarate (α-KG) and NH_4^+, catalyzed by GDH, or α-KG and other amino acids catalyzed by various transaminases. Whether the synthesis of glutamine begins with glutamate or α-KG is determined by the distribution of GS within the cell, and multiple GS genes has been reported for the rainbow trout (Murray et al., 2003; Walsh et al., 2003). Webb and Brown (1976, 1980) determined the distribution of GS in tissues of ureosmotic and non-ureosmotic fishes. They reported the presence of high levels of GS in the cytosolic fraction of brain tissues. However, for ureosmotic fishes, the liver tissues contained high levels of GS activity in the mitochondria. Furthermore, their results (Webb and Brown, 1976, 1980) showed that certain non-ureosmotic fishes possessed cytosolic GS activities in the livers, but the activities were relatively low. GS is also detected from the mitochondria of elasmobranch kidney, which may function as part of a substrate cycle for ammonia excretion during acidosis (King and Goldstein, 1983).

Since NH_3 is an uncoupler of oxidative phosphorylation and is toxic during exit from the matrix of mitochondrion, it is logical for it to be detoxified to a less toxic (or non-toxic) product before being releasing into the blood. For the detoxication of endogenous ammonia, which is produced through transdeamination, to glutamine (or urea), it is essential for GS to be located in the mitochondria. So, glutamate serves the dual functions of providing NH_3 and acting as a direct substrate for glutamine formation. Glutamine formed in the mitochondrial matrix can exit to the cytosol where it serves as a precursor for various biosynthetic pathways. Glutamine shows no protonation of its R chain over a wide range of pHs and is

therefore proton-neutral (Campbell, 1991, 1997). The physiological consequence of this is that NH_3 in the mitochondrial matrix, whether arising by deamination of glutamate via GDH or the direct entry of NH_3 or NH_4^+, exits to the cytosol as a proton-neutral amide-function of glutamine. Through this, the problem of uncoupling oxidative phosphorylation by NH_3 is circumvented.

Ammonia asserts its toxic effects on the brain, and fish brains possess high levels of GS activities to protect them against ammonia toxicity (Peng et al., 1998; Ip et al., 2005b). GS located in the mitochondria would render the detoxification of exogenous ammonia inefficient as NH_3 has to permeate through both the plasma and mitochondrial membranes to be accessed by the mitochondrial GS. NH_3 would bind with H^+ after entering the cytosol to form NH_4^+, and ammonia begins to exert its toxic effects. So, GS in brains of fishes are located in the cytosol; this specific location facilitates the detoxication of ammonia circulated in the blood, and protects the brain from ammonia intoxication. In the case of marine elasmobranchs which depend on urea synthesized through carbamoyl phosphate synthetase III (CPS III) in the liver for osmoregulation, two GS isozymes exist separately in the brain and the liver, localized in the cytosol and the mitochondria, respectively (Smith et al., 1983). In many tropical fishes, high levels of cerebral GS activities correlate well with their high environmental ammonia tolerance. As a result, the brain is often the organ undergoing the largest increases in glutamine content in fish exposed to ammonia.

Certain air-breathing fishes can detoxify endogenous ammonia to glutamine in non-cerebral tissues. Sleepers (the marble goby, *Oxyeleostris marmoratus*, and four-eyed sleeper, *Bostrichthys sinensis*) belonging to the Family Eleotridae and the swamp eel, *Monopterus albus*, belonging to Family Synbranchidae are exceptional because they can detoxify endogenous ammonia to glutamine in their livers and muscles during aerial exposure (Jow et al., 1999; Ip et al., 2001a; Tay et al., 2003) and ammonia-loading (Anderson et al., 2002; Ip et al., 2004e; Chew et al., 2005a). In *B. sinensis*, virtually all the glutamine synthetase activity

in the liver are located in the cytosol, and it can be up-regulated through increased expression of the gene during ammonia exposure (Anderson et al., 2002). Glutamine synthesized is stored within the body, and it can be used for other anabolic processes (e.g., syntheses of purine, pyrimidine, mucopolysaccharides, etc.) when the environmental conditions become more favourable. Unlike urea, it is not an excretory end-product of ammonia detoxification. The Gulf toadfish *Opsanus beta* is another unique example; it detoxifies endogenous ammonia to glutamine to suppress ammonia excretion during confinement stress (Walsh and Milligan, 1995). Interestingly, in mammalian liver, GS is also present in the cytosolic compartment of perivenule hepatocytes (Wu, 1963) and appears to function in these cells as a 'fail-safe' mechanism for ammonia detoxication when the capacity for urea synthesis is exceeded.

3.3.6. Detoxification of ammonia to urea

Differences in water and ionic regulation in seawater as opposed to freshwater may have rendered ammonotely disadvantageous in the marine environment (Campbell, 1973). However, it would be advantageous to accumulate urea as an osmolyte, and the function of the ornithine-urea cycle (OUC) in synthesizing urea for osmoregulatory purposes is seen today in elasmobranchs, holocephans and coelacanths. Marine elasmobranchs are ureogenic because they possess a functional OUC in mitochondria of their livers and muscles (Anderson, 2001; Steele et al., 2005). They are also ureotelic, and urea is the primary product (>50%) of nitrogen excretion. In order to be able to retain urea for osmoregulation, the effective urea permeability in these cartilaginous fishes is decreased. This is achieved as a result of the presence of specific secondarily active (Na^+ coupled) urea transporters in gills and kidney and modification of lipid composition of gills to achieve higher cholesterol-to-phospholipid ratios (Fines et al., 2001; Walsh and Smith, 2001).

In ureogenic fishes, the OUC consists of the enzyme CPS III, ornithine transcarbamylase (OTC), argininosuccinate synthetase, argininosuccinate lyase and arginase. The primary ammonia-fixing enzyme, CPS III, and OTC are located in the matrix of liver mitochondria. CPS III utilizes glutamine as a substrate and therefore ureogenic fishes possess high levels of GS in liver mitochondria. Because NH_3 is already detoxified to glutamine which is proton-neutral, the formation of carbamoyl phosphate via CPS III from glutamine in the liver mitochondrial matrix of fish cannot be regarded as an ammonia-detoxifying system. This is different from higher vertebrates, whose OUC involves carbamoyl phosphate synthetase I (CPS I) which utilizes NH_3 directly as a substrate. For marine elasmobranchs, the localization of GS and CPS III in the liver mitochondrial matrix (Anderson and Casey, 1984) probably evolved more as a mechanism for urea synthesis for osmoregulatory purposes (Ip et al., 2005e; Chew et al., 2006). In this way, glutamine is utilized directly for carbamoyl phosphate, and ultimately urea synthesis, instead of exiting the mitochondria to act as a substrate for other anabolic purposes in the cytosol. Working together, GS, CPS III and OTC form citrulline in the matrix, which then exits the mitochondria. Citrulline is converted to arginine via argininosuccinate synthetase and argininosuccinate lyase in the cytosol. Unlike mammals, arginase is located in the mitochondrial matrix of fish (except lungfishes; Mommsen and Walsh, 1991); so, arginine has to re-enter the matrix where it is converted to urea, regenerating ornithine for citrulline synthesis (Mommsen and Walsh, 1991). Like glutamine, citrulline is proton-neutral (Campbell, 1991, 1997); so, uncoupling of oxidative phosphorylation would not occur.

For those fishes which are ureogenic but non-ureosmotic, the OUC in the liver can apparently detoxify ammonia, which is produced at a high rate mainly in the mitochondrial matrix through the catabolism of excess amino acids from food. There are increases in rates of urea synthesis and excretion in the giant mudskipper *P. schlosseri* (Ip et al., 2004c) and the slender lungfish *Protopterus dolloi* (Lim et al., 2004) within 24 h after feeding. Recent works

on marine sharks (Wood et al., 2005c) and freshwater stingray (Chew et al., 2006) confirm that ureogenic elasmobranchs also increase the rate of urea synthesis after feeding, but urea is retained for the purpose of osmoregulation instead of being excreted.

Only a few teleosts are ureotelic in water (e.g. the gulf toadfish *Opsanus beta* under confined or crowded conditions; Walsh et al. 1990), or able to detoxify a minor quantity of ammonia to urea during ammonia-loading (*Mugilogobius abei*; Iwata et al., 2000). The majority of tropical teleosts studied so far do not use ureogenesis as a major strategy to detoxify endogenous (during aerial exposure) or exogenous and endogenous ammonia (during ammonia loading). These include the mudskippers *Periophthalmodon schlosseri*, *Boleophthalmus boddaerti* and *Periophthalmus modestus* (Iwata and Deguichi, 1995; Peng et al., 1998; Lim et al., 2001), the marble goby *O. marmoratus* (Jow et al., 1999), the four-eyed sleeper *B. sinensis* (Ip et al., 2001a; Anderson et al., 2002), the oriental weatherloach *Misgurnus anguillicaudatus* (Chew et al., 2001; Tsui et al., 2002), the mangrove killifish *Rivulus marmoratus* (Frick and Wright, 2002), the small snakehead *Channa asiatica* (Chew et al., 2003c) and the swamp eel *M. albus* (Tay et al., 2003; Ip et al., 2004e) exposed to terrestrial conditions or ammonia loading for various periods. Ureogenesis in fishes is energetically intensive. For teleosts and elasmobranchs, a total of 5 mol of ATP are hydrolyzed for each mole of urea synthesized, equivalent to 2.5 mol ATP used for each mole of nitrogen assimilated (Ip et al., 2001b). It is probably because of this that ureogenesis is not commonly adopted as a single major strategy to handle ammonia toxicity when air–breathing tropical teleosts (Chew et al., 2006) are exposed to terrestrial conditions or environmental ammonia.

To date, the only teleost for which unequivocal evidence is available for the OUC to function primarily for the purpose of ammonia detoxication is the tilapia *Alcolakia grahami* in Lakes Magadi and Nakuru in Kenya. It thrives in a highly alkaline environment (pH 10), in which NH_3 excretion would be totally impeded. As a result, it develops a high capacity to

detoxify endogenous ammonia to urea via the OUC (Walsh et al., 1993). In fact, this is the first known example of complete ureotely in an entirely aquatic teleost fish (Randall et al., 1989). *A. grahami* contains in its liver significant levels of OUC enzymes and considerable GS activity. In addition, CPS III and all other OUC enzyme activities are present in the muscle at levels more than sufficient to account for the rate of urea excretion (Lindley et al., 1999). It is highly unusual that the muscle CPS can use NH_3 as a substrate; but, because of this adaptation, there is no need for GS and OUC to be tightly coupled, and GS is not well expressed in muscle (Lindley et al., 1999). So, like the OUC in mammals, NH_3 is directly converted via CPS and OTC to citrulline which exits the muscle mitochondria of *A. grahami* without uncoupling oxidative phosphorylation (Campbell, 1997). This is an important development for OUC to function primarily for ammonia detoxication, so that ammonia can be excreted as urea without being affected by the ambient alkaline water. The majority of the urea synthesized in *A. grahami* is excreted through the gills (Wood et al., 1994).

In contrast, African lungfishes, which belong to Class: Sarcopterygii (lobe-finned fishes), synthesize and accumulate urea during emersion and aestivation on land. African lungfishes are ureogenic and they possess a full complement of OUC enzymes (Janssens and Cohen, 1968a; Mommsen and Walsh, 1989), including CPS III, in their livers (Chew et al., 2003b; Loong et al., 2005). Chew et al. (2004) demonstrated that the slender lungfish, *P. dolloi*, detoxified endogenous ammonia to urea, and the rate of urea synthesis increased 2.4- and 3.8-fold during 6 and 40 days (equivalent to 12 and 46 days, respectively, in this study), respectively, of aestivation in air. Why then African lungfish would detoxify ammonia to urea? When urea is injected intra-peritoneally into *P. dolloi*, only a small percentage (34%) of it is excreted during the subsequent 24-h period (Ip et al., 2005d). At hour 24, significant quantities of urea are retained in various tissues of *P. dolloi*, which lead to an apparent reduction in endogenous ammonia production, a significant decrease in the hepatic arginine content, and a significantly lower level of brain tryptophan in this lungfish (Ip et al., 2005d).

All these three phenomena have been observed previously in aestivating *P. dolloi* (Chew et al., 2004). So, increased urea synthesis and accumulation may have a physiological role in initiating and perpetuating aestivation in this lungfish. Moreover, accumulation of urea in an aestivating lungfish may reduce the rate of evaporative water loss through vapour pressure depression.

3.3.7. High tissue ammonia tolerance, especially in the brain

Once ammonia is released into the blood, it will exert toxic effects on other cell types. The two vital organs with excitable cell types are the heart and the brain. However, at least for rainbow trout, the heart does not seem to be the organ where ammonia toxicity acts (Tsui et al., 2004), and that leaves the brain as the main target of ammonia toxicity in fish. Ammonia apparently enters the brain by diffusion and not through transporters (Cooper and Plum, 1987). It has been shown that the blood brain barrier permeability for $^{13}NH_4^+$ is only ~0.5% that of $^{13}NH_3$ in Rhesus monkey (Raichle and Larson, 1981). This implies that the passage of ammonia across the blood brain barrier depends partly on the arterial blood pH (Cooper and Plum, 1987). Once ammonia gets through the blood brain barrier, it can permeate the plasma membrane of brain cells as NH_3.

In mammals, high levels of brain ammonia (1-3 mmol l^{-1}) lead to glutamatergic dysfunction (Rose, 2002; Felipo and Butterworth, 2002) which remains as the leading candidate in the pathogenesis of hepatic encephalopathy in acute liver failure. However, many tropical fishes (see Ip et al., 2004b, Chew et al., 2006 for reviews) can tolerate high levels of environmental ammonia, and the environmental tolerance correlates well with their high tolerance of ammonia at the cellular and sub-cellular levels (Ip et al., 2005a). This adaptation facilitates the accommodation of relatively high concentrations of ammonia in the blood, which can reduce the net influx of NH_3 by lowering the inwardly directed ΔP_{NH3} during ammonia loading. In addition, a buildup of ammonia in the body may be a pre-

requisite for volatilization of NH_3 in certain air-breathing fish species (Tsui et al., 2004). The blood brain barrier of fish is apparently permeable to NH_3, because the brain ammonia content of certain fish species can build up to very high levels under certain conditions. The mechanisms involved in ammonia tolerance in the brain cells of these ammonia-tolerant fishes are presently uncertain. However, it is evident that their brains detoxify the permeated ammonia to glutamine (Chew et al., 2005a), although glutamine accumulation and consequential astrocyte swelling is known to be one of the reasons behind hepatic encephalopathy in mammals (Brusilow, 2002).

Ip et al. (2005b) studied chronic and acute ammonia intoxication in mudskippers, *P. schlosseri* and *B. boddaerti*. For *P. schlosseri* and *B. boddaerti* exposed to sublethal concentrations (100 and 8 mmol l^{-1}, respectively, at pH 7.0) of environmental ammonia, brain ammonia contents increased drastically during the first 24 h, reaching 18 and 14.5 μmol g^{-1}, respectively, at hour 96. Simultaneously, glutamine accumulated to exceptionally high levels in the brains of these two mudskippers (14-18 μmol g^{-1}) without causing death. So, these mudskippers can ameliorate problems associated with glutamine synthesis and accumulation as observed in mammals (Ip et al., 2005b). *P. schlosseri* and *B. boddaerti* can also tolerate high doses of ammonium acetate injected into their peritoneal cavities, with 24 h LC_{50} of 15.6 and 12.3 μmol g^{-1} fish, respectively (Ip et al., 2005b). After the injection with a sublethal dose of ammonium acetate, there were significant increases in ammonia and glutamine levels in their brains at hour 0.5, but these levels returned to normal at hour 24. In contrast, for *P. schlosseri* and *B. boddaerti* that succumbed within 15-50 min to a LC_{50} dose of ammonia acetate, the ammonia contents in the brains reached much greater levels (12.8 and 14.9 μmol g^{-1}, respectively), while the glutamine level remained relatively low (3.93 and 2.67 μmol g^{-1}, respectively). Thus, unlike mammals (Brusilow, 2002), glutamine synthesis and accumulation in the brain is not the major cause of death in these two mudskippers confronted with acute ammonia toxicity. Indeed, the GS inhibitor methionine sulfoximine

(MSO), at a dosage (100 μg g^{-1} fish) protective for mammals, does not protect *B. boddaerti* against acute ammonia toxicity, although it is an inhibitor of GS activities from the brains of both mudskippers (Ip et al., 2005b). In the case of *P. schlosseri*, MSO only prolongs the time to death but does not reduce the mortality rate (100%). Using magnetic resonance imaging, Veauvy et al. (2005) demonstrated that the increase in brain glutamine was not associated with any changes in brain water in the Gulf toadfish, *O. beta*, exposed to ammonia. Again, the lack of brain water accumulation implies that ammonia exerts its toxic effects via pathways other than cerebral swelling in toadfish. Furthermore, toadfish pre-treated with MSO do not survive a normally sub-lethal exposure to 3.5 mmol l^{-1} ammonia. This suggests that detoxification of ammonia to glutamine catalyzed by GS is critical to ammonia tolerance in this fish.

In addition, (5R, 10S)-(+)-methyl-10, 11-dihydro-5H-dibenzo[a, d]cyclohepten-5, 10-imine hydrogen maleate (MK801), which is an antagonist of NMDA receptors, at a dosage of 2 μg g^{-1} fish has no protective effect on *P. schlosseri* and *B. boddaerti* injected with a lethal dose of ammonium acetate, indicating that activation of NMDA receptors is not the major cause of death during acute ammonia intoxication (Ip et al., 2005b). Thus, unlike mammals (Marcaida et al., 1992; Kosenko et al., 2000), activation of NMDA receptors may not be the explanation for acute ammonia toxicity in the brains of *P. schlosseri* and *B. boddaerti*. Indirectly, these results are in support of the proposition that astrocyte swelling may not have occurred in the brains of these two mudskippers. This is because NMDA receptors are activated by extracellular glutamate, and astrocyte swelling can lead to an increase in extracellular glutamate concentration under cell-culture conditions (Kimelberg et al., 1990) due to an increase in glutamate release and/or decrease in glutamate uptake. In animals, an increase in intracellular NH_4^+ would lead to changes in membrane potential (Sugden and Newsholme, 1975) which would result in the reversal of glutamate transport and hence an increase in the extracellular glutamate concentration (Szatkowski et al., 1990). In addition,

membrane depolarization can lead to the removal of the Mg^{2+} block on NMDA receptors and result in their activation (Fan and Szerb, 1993). Hence, it would appear that *P. schlosseri* and *B. boddaerti* have special abilities to control the intracellular ammonia level in their brains despite drastic increases in brain ammonia contents (intracellular + extracellular). NH_4^+ can replace K^+ in the facilitated diffusion of K^+ through K^+ channels and/or active transport of K^+ through Na^+, K^+-ATPase; both these processes have direct or indirect deleterious effects on the membrane potential of a cell. In view of the high levels of ammonia in the brains of *P. schlosseri* and *B. boddaerti* exposed to chronic and acute ammonia toxicity and the lack of protective effect from MK801, it can be deduced that either membrane depolarization occurred but did not lead to activation of NMDA receptors, or membrane potentials were resilient to NH_4^+ interference due to the presence of K^+ channels and Na^+, K^+-ATPase with high substrate specificities for K^+, in the brains of these two mudskippers.

Wee et al. (2007) examined the mechanism of acute ammonia toxicity in the African sharptooth catfish *Clarias gariepinus* and evaluated whether [MSO; an inhibitor of glutamine synthetase (GS)] or MK801 [an antagonist of N-methyl D-aspartate type glutamate (NMDA) receptors] had a protective effect against acute ammonia toxicity in this fish. After 48 h of exposure to a sublethal concentration (75 mmol l^{-1}) of environmental ammonia, the brain glutamine and ammonia contents in *C. gariepinus* increased to 15 μmol g^{-1} and 4 μmol g^{-1}, respectively. Thus, *C. gariepinus* detoxified ammonia to glutamine and could tolerate high levels of glutamine in its brain. After *C. gariepinus* was injected intraperitoneally with a sublethal dose of ammonium acetate (CH_3COONH_4; 8 μmol g^{-1} fish) followed with emersion, brain ammonia and glutamine contents increased continuously during the subsequent 24-h period, reaching 7 and 18 μmol g^{-1}, respectively, at hour 24. These results suggest that when confronted with acute ammonia toxicity, the survival of *C. gariepinus* was crucially determined by its high tolerance of ammonia and high capacity to detoxify ammonia to glutamine in the brain. For fish injected with a sublethal dose of CH_3COONH_4 (10 μmol

g^{-1} fish) followed with immersion, there were transient but significant increases in brain ammonia and glutamine contents, which peaked at hour 2 (4 μmol g^{-1}) and hour 6 (6 μmol g^{-1}), respectively. From these results, it can be deduced that *C. gariepinius* accumulated glutamine in preference to ammonia in its brain. By contrast, for fish injected with a lethal dose (20 μmol g^{-1} fish) of CH_3COONH_4 followed with immersion, the brain ammonia content increased drastically to 10 μmol g^{-1} after 30 min, while the brain glutamine content remained relatively low at 5 μmol g^{-1}. Therefore, it can be concluded that increased synthesis and accumulation of glutamine in the brain was not the major cause of death in *C. gariepinus* confronted with acute ammonia toxicity. The determining factor of acute ammonia toxicity appeared to be the rate of ammonia build-up in the brain. MK801 (2 μg g^{-1} fish) had no protective effect on *C. gariepinus* injected with a lethal dose of CH_3COONH_4 (20 μmol g^{-1} fish) indicating that activation of NMDA receptors might not be involved. By contrast, the prior administration of MSO (100 μg g^{-1} fish) reduced the mortality rate from 100% to 80% and at the same time prolonged the time of death significantly from 27 min to 48 min. However, the protective effect of MSO was apparently unrelated to the inhibition of glutamine synthetase and prevention of glutamine accumulation in the brain. Instead, MSO affected activities of glutamate dehydrogenase and alanine aminotransferase and suppressed the rate of ammonia build up in the brain of fish injected with a lethal dose of CH_3COONH_4.

3.4. Lungfishes, with emphases on African species

3.4.1. Six species of extant lungfishes belonging to three Families

The Dipnoi are an archaic group of fishes belonging to the Class Sarcopterygii, and are characterized by the possession of a lung opening off the ventral side of the oesophagus. Members of three different Families: Protopteridae (*Protopterus dolloi*, *Protopterus amphibicus*, *Protopterus aethiopicus* and *Protopterus annectens*), Lepidosirenidae (*Lepidosiren paradoxa*), and Ceratodontidae (*Neoceratodus forsteri*) are found in Africa,

South America and Australia, respectively. They are called lungfish in general. The gills of the African and South American lungfish are reduced and inadequate to reply on them completely for respiration.

The fish-tetrapod transition represents one of the greatest events in vertebrate evolution. Air breathing evolved in fish (e.g. lungfishes), but prolonged terrestrial respiration is a tetrapod feature. Similarly, limbs with strong skeletal units appeared in Sarcopterygian fishes, but the loss of fin rays and appearance of digits are features of tetrapods (Forey, 1986). Lungfishes depend entirely on aerial respiration and can live for an extended period out of water. There are few similarities between lungfishes and tetrapods, particularly amphibians, in aspects of gas exchange and excretory physiology, pulmonary circulation, and heart structure (Forey et al., 1991; Schultze, 1994). Though unable to move about to any great extent on land, lungfishes, with the exception of the Australian *N. forsteri*, can live for an extended period out of water.

3.4.2. Only African lungfishes can aestivate in arid conditions at high temperature

The African lungfishes are usually found in marginal swamps and backwaters of rivers and lakes. During the dry season, they (*Protopterus aethiopicus* and *Protopterus annectens*) aestivate in subterranean mud cocoons or in a layer of dried mucous on land (*Protoperus dolloi*) (Poll, 1961). They can exist in this state for over a year, although normally they aestivate only from the end of one wet season to the start of the next. African lungfish have been kept alive in aestivation in the laboratory for 3 years (Smith, 1930), and Smith calculated that they have the metabolic resources to survive for 5 years. They can drastically reduce the rate of ammonia production during aestivation (Janssens and Cohen, 1968a, b). The South American lungfishes live in the Amazon River basin and Paraguay-Parana river basin. They prefer stagnant waters where there is little current. During the dry

period, they burrow into the mud, to a depth of about 30-50 cm, and seal off the entrance with clay, leaving 2-3 holes for respiration. Metabolism is reduced during this period of aestivation. The Australian lungfishes can be found in still or slow-flowing waters, usually in deep pools. During periods of drought, it can tolerate stagnant conditions by breathing air. However, it lacks the ability to survive dry spells by aestivation.

3.4.3. Urea synthesis and CPS in African lungfishes

African lungfishes have a greater OUC capacity (Janssens, 1964; Janssens and Cohen, 1966; Forster and Goldstein, 1966) than their non-aestivating Australian counterpart (Goldstein et al., 1967). On land, there is a lack of water to flush the branchial and cutaneous surfaces, impeding the excretion of ammonia, and consequently leading to the accumulation of ammonia in the body. Ammonia is toxic (Ip et al., 2001b) and therefore African lungfishes have to avoid ammonia intoxication when out of water. Previous works on the *P. aethiopicus* and *P. annectens* reveal that they are ureogenic (Janssens and Cohen, 1966; Mommsen and Walsh, 1989). Similar to tetrapods, they possess mitochondrial CPS I, which utilize NH_4^+ as a substrate, and an arginase which is present mainly in the cytosol, of the liver (Mommsen and Walsh, 1989). On the other hand, coelacanths, marine elasmobranchs and some teleosts are known to have CPS III (Mommsen and Walsh, 1989; Anderson, 1980; Randall et al., 1989), which utilizes glutamine as a substrate, and an arginase in the hepatic mitochondria. It has been suspected that the replacement of CPS III with CPS I, and mitochondrial arginase with cytosolic arginase, occurred before the evolution of the extent lungfishes (Mommsen and Walsh, 1989).

However, Chew et al. (2003b) demonstrated that, like coelacanths, elasmobranchs and some teleosts, *P. dolloi* possesses CPS III in the liver, and not CPS I as has been shown previously in other African lungfishes. Yet, similar to tetrapods, hepatic arginase is present

mainly in the cytosol. Since *P. dolloi* possessed CPS III, then it would be essential for it to have GS in the hepatic mitochondria to supply the glutamine needed for urea synthesis *de novo*. Indeed, GS activity is present in both the mitochondrial and cytosolic fractions of the liver of *P. dolloi*. Therefore, it would be essential to re-examine the type of CPS present in other African lungfishes (e.g. *P. annectens* and *P. aethiopicus*) to confirm that they indeed possess CPS I. If they actually possess CPS III, then the participation and the role of lungfishes in the evolution of CPS III to CPS I (Mommsen and Walsh, 1989) must be re-evaluated.

3.4.4. Excretory nitrogen metabolism in the African lungfishes

3.4.4.1. Aerial exposure

The slender lungfish, *P. dolloi*, is a lungfish found in Central Africa in the lower and middle regions of the Congo River basin. *Protopterus dolloi* retains the capacity to aestivate when out of water, although unlike other African lungfishes, it does not have to aestivate inside a subterranean cocoon. During aerial exposure, the ammonia excretion rate in *P. dolloi* decreases significantly to 8-16% of the submerged control, because of a lack of water to flush the branchial and cutaneous surfaces (Chew et al., 2003b). However, there are no significant increases in ammonia contents in the muscle, liver, brain or plasma exposed to air for 6 days. In addition, the rate of ammonia excretion of the experimental animal remains low and does not return to the control level during the subsequent 24-h period of re-immersion. These results suggest that (1) endogenous ammonia production is drastically reduced and (2) endogenous ammonia is detoxified effectively into urea. Indeed, there are significant decreases in glutamate, glutamine and lysine levels in the liver of fish exposed to air, which lead to a decrease in the total free amino acid (TFAA) content. This indirectly confirms that the specimen has reduced its rates of proteolysis and/or amino acid catabolism

to suppress the production of ammonia. *Protopterus dolloi* also reduces ammonia production during 40 d of aestivation in a mucus cocoon on land (Chew et al. 2004). In addition, Chew et al. (2004) obtained results suggesting that *P. dolloi* was capable of reducing ammonia production during ammonia loading.

Since *P. dolloi* aestivates within a dry layer of mucus on land (Brien, 1959; Poll, 1961) instead of in a cocoon inside the mud like *P. aethiopicus* and *P. annectens*, it is likely that African lungfishes evolved through a sequence of events, i.e., air breathing, migrate to land, and then burrow into mud. Aestivation can occur on land or in mud, but the latter must have certain advantages over the former, for instance, avoidance of predation. Therefore, Chew et al. (2003b) speculated that burrowing into the mud could be a more advanced development during evolution.

During 79-128 days of aestivation out of water, *P. aethiopicus* accumulates urea in its body (Janssens and Cohen, 1968a). However, it was reported that urea accumulation did not involve an increased rate of urea synthesis (Janssens and Cohen, 1968a), even though the animals appear to be in continuous gluconeogenesis throughout aestivation (Janssens and Cohen, 1968b). This apparent controversy arose because of two counteracting factors: (1) increase in the rate of urea production, and (2) decrease in the rate of ammonia production. During the initial phase of aerial exposure before the onset of a reduction in the rate of ammonia production, the rate of urea synthesis *de novo* theoretically has to be increased to detoxify ammonia which is produced at a normal (or slightly sub-normal) rate and cannot be excreted. After entering into aestivation for a relatively long period, ammonia production rate would have been suppressed (Smith, 1935; Janssens, 1964). This would subsequently result in a decrease in the rate of urea synthesis *de novo*, leading to those observations made in previous studies (Janssens and Cohen, 1968 a, b). This analysis led Chew et al. (2003b) to

hypothesize that the rate of urea synthesis would increase in *P. dolloi* exposed to air without undergoing aestivation.

There are significant increases in the urea levels in the muscle (8-fold), liver (10.5-fold), and plasma (12.6-fold) of specimens exposed to terrestrial conditions for 6 days. Furthermore, there is a significant increase in the urea excretion rate in specimens exposed to terrestrial conditions for 3 days or more (Chew et al., 2003b). Taken together, it would mean *P. dolloi* increases the rate of urea synthesis during this 6-days period of aerial exposure. This is supported by the fact that aerial exposure leads to an increase in the hepatic OUC capacity, with significant increases in the activities of CPS III (3.8-fold), argininosuccinate synthetase + lyase (1.8-fold) and more importantly GS (2.2-fold), in *P. dolloi* (Chew et al., 2003b).

Upon re-immersion, the urea excretion rate in *P. dolloi* increases 22-fold as compared to the control specimen (Chew et al., 2003b), which is probably the greatest increase amongst fishes. These results suggest that, unlike marine elasmobranchs, *P. dolloi* probably possesses mechanisms which facilitate the excretion of urea in water, and that these mechanisms, in contrast to those of metamorphosed amphibians, does not function well on land.

3.4.4.2. Aestivation

Chew et al. (2004) also studied the strategies adopted by *P. dolloi* to ameliorate the toxicity of endogenous ammonia during short (6 days) or long (40 days) periods of aestivation in a layer of dried mucus in air in the laboratory. Despite decreases in rates of ammonia and urea excretion, the ammonia contents in the muscle, liver, brain or gut of *P. dolloi* remain unchanged after 6 days of aestivation. For specimens aestivated for 40 days, the ammonia contents in the muscle, liver and gut of specimens decrease significantly instead, which suggest the occurrence of a decrease in the rate of ammonia production.

However, contrary to former reports on *P. aethiopicus* (Janssens and Cohen, 1968a, b), there is a significant increase in the rate of urea synthesis in *P. dolloi* during 40 days of aestivation (Chew et al., 2004). The excess urea formed is mainly stored in the body.

3.4.4.3. Exposure to environmental ammonia

There is no doubt that the capability of detoxifying ammonia to urea contributes to the lungfishes' success in aestivating on land. However, there is a dearth of information on the response of lungfishes in general to ammonia loading in the literature. In nature, *P. dolloi* encounters aerial exposure occasionally during drought. However, before the water totally dries up and leads to a reduction in ammonia excretion, the exogenous ammonia would be concentrated to high levels in the external medium, creating an ammonia-loading situation. Consequently, with a reversed ΔP_{NH3} gradient, exogenous ammonia may penetrate the skin and branchial surfaces into the body of the fish. In the laboratory, *P. dolloi* can tolerate high environmental ammonia (HEA), up to 100 mmol l^{-1} NH_4Cl at pH 7 for at least 6 days (Chew et al., 2005b).

In an external medium containing 30 or 100 mmol l^{-1} NH_4Cl at pH 7, both ΔP_{NH3} and NH_4^+ concentration gradients are directed inward. Yet, the plasma ammonia concentrations in *P. dolloi* exposed to these two concentrations of NH_4Cl are very low, and the values (0.288 and 0.289 mmol l^{-1}, respectively) are comparable. So, how does *P. dolloi* maintain such low levels of plasma ammonia despite the large inwardly directed NH_3 and NH_4^+ gradients? This can be achieved in part through the synthesis of urea *de novo* and its subsequent excretion. However, even then, the rate of ammonia removal must be fast enough to balance the rate of endogenous ammonia production and the influx of exogenous ammonia. An analysis of the nitrogen budget in a specimen exposed to 30 mmol l^{-1} NH_4Cl reveals that there is a reduction in ammonia production during the 6-days experimental period (Chew et al., 2005b). In

addition, the rate of urea synthesis is up-regulated to detoxify both the endogenous and net influx of exogenous ammonia, which can be small due to the low NH_3 permeability of its skin (see below) and its being an air-breather (Chew et al., 2005b).

It is because the plasma ammonia concentration is maintained at low levels that a continuous influx of NH_3 into the body of *P. dolloi* would occur. It is likely that *P. dolloi* could afford such a strategy because its body surfaces have low permeability to NH_3. Chew et al. (2005b) estimated the flux of ammonia through the skin of *P. dolloi* down a 10-fold ammonia gradient at pH 7 as 0.003 μmol min^{-1} cm^{-2}, which is even lower than that of the giant mudskipper *P. schlosseri* (0.01 μmol min^{-1} cm^{-2}, Ip et al., 2004d). The branchial epithelial surface of aquatic teleost fishes has a higher permeability to NH_3 due to its major function in gaseous exchange. A reduction of the effective area of the branchial epithelium in *P. dolloi* also contributes to its ability to reduce the influx of exogenous NH_3. Both *Protopterus dolloi* and *Periophthalmodon schlosseri* maintain low internal ammonia levels in HEA (30-100 mmol l^{-1} NH_4Cl), although by different mechanisms, i.e., urea synthesis and active NH_4^+ transport, respectively. Thus, they share the common need to reduce the influx of NH_3 in order to render these mechanisms effective. At present, it is uncertain if *P. dolloi* or any other lungfishes can excrete acid to detoxify NH_3 externally as in *P. schlosseri* and *B. boddaerti*.

3.4.4.4. Feeding versus injection of NH_4Cl and/or urea

Mommsen and Walsh (1991) postulated that since urea-N was much more costly to make than ammonia-N, marine elasmobranchs may excrete extra exogenous nitrogen, over and above the needs of osmoregulation, in the form of ammonia-N rather than urea-N. To date, the only information available to answer this hypothesis comes from a study in which dogfish shark were infused with ammonia at a rate of 1500 μmol kg^{-1} h^{-1} for 6 h (Wood et al.,

1995). Both ammonia-N and urea-N excretion increased by similar extents during infusion, though the former more rapidly, and the entire ammonia-N load (actually 132%) was excreted within 18 h. Hence, Mommsen and Walsh's (1991) hypothesis appears to be correct for marine elasmobranchs. However, since energy consumption is the major issue in whether ammonia would be detoxified to urea, the excretion of infused/injected ammonia as ammonia per se could be favoured simply because of the lack of a simultaneous supply of extra energy resources. Moreover, unlike endogenous ammonia, ammonia infused/injected into the fish does not originate within cells, and has to penetrate through the plasmalemma and mitochondrial membranes in order to be accessible to the OUC enzymes. That means infused/injected ammonia is more likely to be excreted through the gills once it is absorbed into the blood.

Feeding, on the other hand, results in the catabolism of excess amino acids absorbed after food digestion. This would lead to the release of endogenous ammonia in sub-cellular compartments, specifically the hepatic mitochondria, via transdeamination (Campbell, 1991). Consequently, ammonia produced endogenously is more likely to activate the OUC present within liver cells. In addition, the problem of ureogenesis being energy-intensive would be circumvented by an ample supply of energy resources after feeding. Therefore, experiments on feeding might provide results different from those involving the infusion/injection of ammonia into the fish, and would offer new insights into the physiological role of OUC in ureogenic fishes.

Ip et al. (2005d) injected NH_4Cl into the peritoneal cavity of *P. dolloi* in the absence of a supply of food and confirmed that the majority of the injected ammonia was excreted as ammonia per se within 24 h. This apparently contradicted the observations made by Chew et al. (2005b) that *P. dolloi* was able to survive in high concentrations (30 or 100 mmol l^{-1} NH_4Cl) of environmental ammonia by up-regulating both the rates of urea synthesis and its

excretion. The controversy arose because of one obvious reason—the excretion of endogenous ammonia by *P. dolloi* was impeded in the presence of high concentrations of environmental ammonia (Chew et al., 2005b), but was not affected at all when ammonia (as NH_4Cl) was injected into the fish peritoneally. In the latter conditions, the injected ammonia, although it resulted in a momentary increase in the concentration of ammonia in the extracellular fluid, could be excreted easily because of the absence of a reversed ΔP_{NH3} (Ip et al., 2005d). These observations led Ip et al. (2005d) to postulate that urea synthesis in *P. dolloi* could respond to intracellular (endogenous) ammonia concentration more readily than the extracellular ammonia concentration. In addition, they (Ip et al., 2005d) postulated that feeding might lead to an increase in urea synthesis in *P. dolloi*.

Indeed, there are significant increases in the rate of ammonia excretion in *P. dolloi* between hour 6 and hour 15 after feeding (Lim et al., 2004). Simultaneously, there are significant increases in urea excretion rates between hour 3 and hour 18. As a result, there is a significant increase in the percentage of total nitrogen (N) excreted as urea-N, which exceeds 50%, between hour 12 and hour 21 post-feeding (Lim et al., 2004). Therefore, it can be concluded that *P. dolloi* shifts from ammonotely to ureotely momentarily after feeding. At h 12 post-feeding, the accumulation of urea-N is greater than the accumulation of ammonia-N in various tissues, which indirectly suggests that feeding leads to an increase in the rate of urea synthesis in *P. dolloi*. This is different from the results obtained by the injection of NH_4Cl into the peritoneal cavity of this fish; 80% of the injected ammonia is excreted within the subsequent 24 h, a large portion of which was ammonia (Ip et al., 2005d). Feeding is more likely to induce urea synthesis because it provides an amply supply of energy resources and leads to the production of endogenous ammonia intracellularly in the liver. The urea synthetic capacity in *P. dolloi* is apparently adequate to prevent a big surge in plasma ammonia level (Lim et al., 2004) as has been observed in other fishes (Wicks and Randall,

2002a), although the brain of *P. dolloi* is likely to be confronted with ammonia toxicity as indicated by a significant increase in the glutamine content at hour 24 (Lim et al., 2004).

4. Chapter 1

Ornithine-urea cycle and urea synthesis in the African lungfish, *Protopterus annectens*, exposed to terrestrial conditions for 6 days

4.1 Introduction

The appearance of tetrapods was one of the most important events in vertebrate evolution, and the ancestor of tetrapods has long been assumed to be a bony fish belonging to the group Sarcopterygii (Romer, 1966). This group includes the Dipnoi (lungfishes), the Rhipidistia (extinct forms), and the Actinistia (coelacanths). As the extinct rhipidistians are not thought to be a monophyletic group (Forey, 1988), the relationship among sarcopterygians and tetrapods remains very problematical. Three possible hypotheses concerning the relationship among extant groups of sarcopterygians (coelacanths, lungfishes, and tetrapods) have been proposed (Forey, 1988) that respectively regard lungfishes and tetrapods (Miles, 1977; Rosen et al., 1981; Forey, 1987), coelacanths and tetrapods (Miles, 1975; Fritsch, 1987; Schultze, 1987), and lungfishes and coelacanths (Northcutt, 1987; Chang, 1991) as sister groups.

To clarify the relationship among coelacanth, lungfishes, and tetrapods, Yokobori et al. (1994) compared the amino acid sequences deduced from the nucleotide sequences of mitochondrial cytochrome oxidase subunit I genes. Their analyses consistently indicate a coelacanth/lungfish clade, but a coelacanth/lungfish clade has not been widely accepted in previous studies, although some morphologists have reached this conclusion (Northcutt, 1987). Later, Zardoya and Meyer (1996) reported that an analysis of the relatedness of mitochondrial DNA in the coelacanths, lungfishes and tetrapods was in favour of the hypothesis that lungfishes were the closest living relatives of terrestrial vertebrates. However, a re-analysis of the data led Rasmussen et al. (1998) to conclude that lungfishes occupied a basal position among gnathostome fish as the sister-group to all other bony fishes.

As a pre-adaptation to water/land transition, the early tetrapod has to acquire the capacity to synthesize urea through the ornithine-urea cycle (OUC) during periods of restricted water availability. Water shortage impedes the excretion of ammonia, leading to its accumulation, but ammonia is toxic (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip

et al., 2001b; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002; Chew et al., 2006). In tetrapods, the toxicity of ammonia is ameliorated by carbamoyl phosphate (leading to urea) formation, catalysed by carbamoyl phosphate synthetase I (CPS I) in the OUC. CPS I utilizes NH_4^+ as a substrate and is activated by N-acetylglutamate. Previous works on the African lungfishes, *Protopterus aethiopicus* and *Protopterus annectens*, suggested the presence of mitochondrial CPS I and a cytosolic arginase in the liver (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989). By contrast, ureosmotic coelacanths, like elasmobranchs, possess in their livers CPS III, which utilizes glutamine as a substrate, and a mitochondrial arginase (Mommsen and Walsh, 1989, 1991). The previous assertion of the presence of CPS I in lungfishes lends support to a lungfish/tetrapod clade, and suggests that the replacement of CPS III with CPS I, and mitochondrial arginase with cytosolic arginase, occurred before the evolution of extant lungfishes (Mommsen and Walsh, 1989, 1991).

On the contrary, Chew et al. (2003b) demonstrated recently that, like marine elasmobranchs, the slender African lungfish *Protopterus dolloi* possesses CPS III in the liver, and not CPS I as has been shown previously in *P. aethiopicus* and *P. annectens*. In addition the activity of glutamine synthetase, which catalyzes the formation of glutamine (the preferred substrate for CPS III), is present in both the mitochondrial and cytosolic fractions of the liver of *P. dolloi*. Therefore, Chew et al (2003b) postulated that *P. dolloi* was more primitive than *P. aethiopicus* and *P. annectens*. Since previous works on *P. aethiopicus* and *P. annectens* followed different CPS assay methods, it is imperative to re-determine the properties and activities of their CPS using the radioactive method (Anderson and Walsh, 1995) adopted by Chew et al. (2003b) to confirm if they indeed possess CPS I. If they happened to possess CPS III like *P. dolloi*, then the current view of lungfishes possessing CPS I (Mommsen and Walsh, 1989) must be re-evaluated. Thus, the first objective of this study was to determine the type of CPS (i.e., CPS I or CPS III) present in the liver of *P.*

annectens, and the second objective was to elucidate the compartmentalization of arginase in their livers.

African lungfishes depend entirely on aerial respiration (Graham, 1997) and are able to survive prolonged periods of drought through aestivation within a layer of dried mucus on land (Brien, 1959; Poll, 1961; Chew et al., 2004) or in subterranean mud cocoons (Smith, 1935; Janssens, 1964; Janssens and Cohen, 1968a, b). Naturally, during drought, lungfishes would be stranded in puddles of water on land before aestivation. As water dries up, the excretion of ammonia would be impeded, leading to the accumulation of ammonia in the body. Chew et al. (2003b, 2004) reported an increase in the rate of urea synthesis, accompanied with an enhanced OUC capacity, in *P. dolloi* during 6 days of aerial exposure or 40 days of aestivation in a dried mucus cocoon. On the contrary, it was reported that urea accumulation in *P. aethiopicus* undergoing 78-129 days of aestivation did not involve an increased rate of urea synthesis (Janssens and Cohen, 1968a), even though the animals appear to be in continuous gluconeogenesis throughout aestivation (Janssens and Cohen, 1968b). During the initial phase of aerial exposure before the onset of a reduction in the rate of ammonia production, the rate of urea synthesis *de novo* theoretically has to be increased to detoxify ammonia which is produced at a normal (or slightly sub-normal) rate and cannot be excreted. Therefore, the third objective of this study was to elucidate whether *P. annectens* was capable of increasing the rates of urea synthesis during 6 days of aerial exposure without undergoing aestivation. Simultaneously, we aimed to elucidate if there was an increase in the OUC capacity of *P. annectens* after 6 days of aerial exposure as in the case of *P. dolloi* (Chew et al., 2003b).

4.2. Materials and methods

4.2.1. Animals

Specimens of *P. annectens* (Owen 1839), weighing 100-150 g body mass, were imported from Africa through a local fish farm in Singapore. Identification of these two species was performed according to Poll (1961). Specimens were maintained in plastic aquaria filled with dechlorinated water at pH 7.0 and at 25°C in the laboratory under a 12 h:12 h light:dark regime. Water was changed daily. No attempt was made to separate the sexes. Fishes were acclimated to laboratory conditions for at least one month. During the acclimatization period, they were fed frozen bloodworm. Food was withdrawn 96 h before experiments, which allowed sufficient time for the gut to be emptied of all food and waste. All experiments were performed under a 12 h:12 h light:dark regime unless stated otherwise.

4.2.2. Verification of the presence of OUC enzymes and GS

The liver, muscle and gut of *P. annectens* in control conditions were excised quickly and homogenized in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.6), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 1 mmol l^{-1} dithiothreitol (DTT) and 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF). The homogenate was sonicated (110 W, 20 kHz; Misonix Incorporated Farmingdale, NY, USA) three times for 20 sec each, with a 10 sec break between each sonication. The sonicated sample was centrifuged at 10, 000 x*g* and 4°C for 15 min. After centrifugation, the supernatant was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with the extraction buffer without EDTA and PMSF. The filtrate obtained was used directly for enzymes assays. Preliminary results indicated that CPS I or III activities were absent from the muscle and gut of *P. annectens*. For comparison, the livers excised from the marine blue-spotted fan-tailed ray *Taeniura lymma* (obtained from the local wet market) and the mouse *Mus musculus*

(obtained through the Animal Holding Unit of the National University of Singapore) were processed at the same time with the liver of *P. annectens* and the CPS activities assayed by the same batch of chemicals.

CPS (E.C. 2.7.2.5) activity was determined according to the method of Anderson and Walsh (1995). Radioactivity was measured using a Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). Enzyme activity was expressed as μmol [^{14}C]urea formed min^{-1} g^{-1} wet mass.

Ornithine transcarbamoylase (OTC; E.C. 2.1.3.3) activity was determined by combining the methods of Anderson and Walsh (1995) and Xiong and Anderson (1989). Absorbance was measured at 466 nm using a Shimadzu 160 UV VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan). Enzyme activity was expressed as μmol citrulline formed min^{-1} g^{-1} wet mass.

Argininosuccinate synthetase (E.C. 6.3.4.5) and lyases (E.C. 4.3.2.1) (ASS + L) activities were determined together assuming that both were present, by measuring the formation of [^{14}C]fumarate from [^{14}C]aspartate using the method of Cao et al. (1991). Radioactivity was measured using a Wallac 1414 liquid scintillation counter. ASS + L activity was expressed as μmol [^{14}C]fumarate formed min^{-1} g^{-1} wet mass.

Arginase (E.C. 3.5.3.1) was assayed as described by Felskie et al. (1998). Urea was determined colorimetrically according to the method of Anderson and Little (1986) as modified by Jow et al. (1998). Arginase activity was expressed as μmol urea formed min^{-1} g^{-1} wet mass.

GS (E.C. 6.3.1.2) was assayed as transferase activity according to the method of Shankar and Anderson (1985). Its activity was expressed as μmol γ-glutamylhydroxymate formed min^{-1} g^{-1} wet mass.

Cellular fractionation of livers from *P. annectens* exposed to terrestrial conditions for 6 days were performed according to the methods of Anderson et al. (2002). Lactate

dehydrogenase and cytochrome *c* oxidase were used as markers for cytosol and mitochondria, respectively.

4.2.3. Evaluation of the effects of 6 days aerial exposure on nitrogenous excretion and accumulation

Specimens of *P. annectens* were immersed individually in 1.5 l of water at 25°C in separate plastic tanks (L20.5 cm × W14.5 cm × H6 cm). Preliminary experiments on the analysis of ammonia and urea in the water sampled at 6 hour and 24 hour showed that the ammonia and urea excretion rates were linear up to at least 24 hour. Subsequently water samples were collected for ammonia and urea assays after 24 hour. The same individuals were then exposed to terrestrial conditions in plastic tanks containing 30 ml of water. After 24 hour, the fish were sprayed thoroughly with water. The water collected was used for ammonia and urea analyses. The process was repeated for 6 days. The disturbance created by the daily collection and introduction of water prevented the experimental subject from initiating aestivation during this period. After 6 days of aerial exposure, specimens were re-immersed in water for 24 hour to determine the rates of ammonia and urea excretion upon recovery. A separate group of fish immersed in water for the same period of time served as the control. Ammonia and urea in water samples were determined according to the methods of Jow et al. (1999).

At the end of 6 days, fish were killed with a strong blow to the head. The lateral muscle, liver and brain were quickly excised. The excised tissues and organs (<1 g) were immediately freeze-clamped in liquid nitrogen with pre-cooled tongs. Frozen samples were kept at -80°C. A separate group of fish exposed to similar conditions was used for the collection of blood samples. The blood was collected in heparinized capillary tubes by caudal puncture. The collected blood was centrifuged at 4,000 x*g* at 4°C for 10 min to obtain the plasma. The plasma was deproteinized in equal volume (v/v) of ice-cold 6%

trichloroacetic acid (TCA) and centrifuged at 10,000 x*g* at 4°C for 15 min. The resulting supernatant was kept at -80°C until analysis.

The frozen samples were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% TCA at 24,000 revs min^{-1} for 20 sec each using an Ultra-Turrax homogenizer with intervals of 10 sec between each homogenization. The homogenate was centrifuged at 10,000 xg and 4^{o}C for 15 min, and the supernatant obtained was kept at -80^{o}C until analysis.

For ammonia analysis, the pH of the deproteinized sample was adjusted to between 5.5 and 6.0 with 2 mol l^{-1} $KHCO_3$. The ammonia content was determined using the method of Bergmeyer and Beutler (1985). The change in absorbance at 25°C and 340 nm was monitored using a Shimadzu UV-160A spectrophotometer. Freshly prepared NH_4Cl solution was used as the standard for comparison.

The urea contents in 0.2 ml of the neutralised sample were analyzed colorimetrically according to the method of Anderson and Little (1986) as modified by Jow et al. (1999). The difference in absorbance obtained from the sample in the presence and absence of urease (Sigma Chemical Co., St. Louis, Missouri, USA, #U7127) was used for the estimation of urea content in the sample. Urea obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) was used as a standard for comparison. Results were expressed as μmol g^{-1} wet mass or mmol l^{-1} plasma.

For FAA analysis, the supernatant obtained was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). FAAs were analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. In spite of a complete analysis of all the FAAs present in the sample, only the contents of those FAAs, which showed significant

changes, and the content of total FAA (TFAA) were reported. Results were expressed as μmol g^{-1} wet mass.

4.2.4. Elucidation of whether the OUC capacity would be enhanced by aerial exposure

Specimens were exposed to the control (immersed) or terrestrial conditions individually in plastic aquaria as described above. OUC enzymes and GS activity in the liver were assayed according to the above-mentioned methods.

4.2.5. Statistical analyses

Results were presented as means ± standard errors of the mean (S.E.M). Student's t-test and one-way analysis of variance followed by Student-Neuman-Keul's multiple range test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $p<0.05$.

4.3. Results

4.3.1. Types of CPS

A full complement of OUC enzymes was present in the liver of *P. annectens* (Table 4.1). The CPS activity from the liver of *P. annectens* had properties similar to that of the blue spotted fan-tail ray *T. lymma* but dissimilar to that of the mouse *M. musculus* (Table 4.1). They utilized mainly glutamine as a substrate, were activated by N-acetylglutamate (NAG), and were refractory to UTP inhibition. No CPS III activities were detectable (detection limit= 0.001 µmol min^{-1} g^{-1}) from the muscle or the gut of *P. annectens*. GS activity was present in the liver, but not in the muscle (Table 4.1).

4.3.2. Compartmentalization of CPS and arginase

After fractionation, the mass specific activities (µmol min^{-1} g^{-1} wet mass) of cytochrome oxidase present in the homogenate, cytosolic and mitochondrial fractions were 0.54 ± 0.05, 0.075 ± 0.011 and 0.71 ± 0.001, respectively. Only 1.2% of lactate dehydrogenase activity was present in the mitochondrial fraction of the liver of *P. annectens*, the activities (µmol min^{-1} g^{-1} wet mass) in the homogenate, cytosolic and mitochondrial fractions being 56 ± 5, 42 ± 8 and 0.68 ± 0.14, respectively. CPS III was present exclusively in the liver mitochondria, but at least 76% of the arginase was present in the cytosol, with activities (µmol min^{-1} g^{-1} wet mass) of 136 ± 25 compared with 178 ± 16 in the homogenate and 4.3 ± 0.7 in the mitochondrial fraction. GS activity (µmol min^{-1} g^{-1} wet mass) was detected in both the cytosolic (0.59 ± 0.09) and mitochondrial (0.41 ± 0.07) fractions of *P. annectens*.

4.3.3. Effects of 6 days of aerial exposure without aestivation on nitrogen metabolism in P. annectens

Six days of aerial exposure led to significant increases in the activities of hepatic GS (2.9-fold) and OTC (1.4-fold) in *P. annectens* after 6 days of aerial exposure (Table 4.1). During 6 days of aerial exposure, the rate of ammonia excretion in *P. annectens* decreased to approximately one-quarter of the control (immersed) value, but returned to control level upon re-immersion on day 7 (Fig. 4.1a). Aerial exposure significantly decreased the rate of urea excretion (≤ 2-fold) on day 3 and 4 (Fig. 4.1b). Upon re-immersion after 6 days of aerial exposure, there was also a 10-fold increase in the urea excretion rate (Fig. 4.1b).

There were no significant changes in the ammonia contents in the muscle, liver, plasma and brain of *P. annectens* (Table 4.2) exposed to terrestrial conditions for 6 days. However, the urea contents in the liver, plasma and brain of *P. annectens* increased 2.7-, 2.8-, and 3.9-fold, respectively, with the urea content in the muscle remained unchanged (Table 4.2).

There was no significant change in the content of TFAA in the muscle of *P. annectens* exposed to terrestrial condition for 6 days, in spite of significant increases in glutamate, glutamine, leucine and threonine contents (Table 4.3). As for the liver, there was a significant decrease in proline content with the TFAA content remained relatively unchanged (Table 4.3).

Table 4.1. Mass specific activities (μmol min^{-1} g^{-1} wet mass) of glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase + lyase (ASS + L) and arginase from the livers of Mus musculus (mouse), Taeniura lymma (stingray), and Protopterus annectens (lungfish), and effects of 6 days of aerial exposure on activities of these enzymes in the livers of *Protopterus annectens.*

	Mass specific activities			
	M. musculus	*T. lymma*	*P. annectens*	
			Immersed	Terrestrial
GS	87±5	13±2	0.36 ± 0.04	1.03 ± 0.19 *
CPS				
Glutamine	n.d.	0.29±0.04	n.d.	n.d.
Glutamine + NAG	n.d.	0.76±0.10	0.061 ± 0.008	0.125 ± 0.027
Glutamine + NAG + UTP	n.d.	0.73±0.11	0.061 ± 0.006	0.125 ± 0.027
Ammonia	n.d.	0.011±0.009	0.002 ± 0.001	0.002 ± 0.001
Ammonia + NAG	4.6±0.4	0.11±0.006	0.002 ± 0.001	0.003 ± 0.001
Ammonia + NAG + UTP	4.4±0.2	0.055±0.002	0.001 ± 0.001	0.002 ± 0.001
OTC	93±5	26±2	13 ± 0.7	18 ± 2 *
ASS + L	1.0±0.1	0.64±0.12	0.27 ± 0.03	0.448 ± 0.079
Arginase	182±17	104±22	113 ± 13	145 ± 30

Results represent mean ± S.E.M (N=4).

NAG, N-acetyl-L-glutamate; UTP, uridine triphosphate

n.d., not detectable (Detection limit = 0.001 μmol min^{-1} g^{-1} wet mass)

*Significantly different from the corresponding immersed condition (p<0.05)

Table 4.2. Effects of 6 days of aerial exposure on contents (μmol g^{-1} wet mass or μmol ml^{-1}) of ammonia and urea in the muscle, liver, plasma and brain of *Protopterus annectens*.

	Ammonia		Urea	
Tissue	Immersed	Terrestrial	Immersed	Terrestrial
Muscle	0.21 ± 0.09	0.28 ± 0.09	3.30 ± 1.03	8.23 ± 1.94 (3)
Liver	0.35 ± 0.11	0.33 ± 0.02	4.79 ± 2.44	13.1 ± 1.3 (3) *
Plasma	0.23 ± 0.02 (3)	0.24 ± 0.02 (3)	4.01 ± 1.46	11.3 ± 1.8 (3) *
Brain	0.052 ± 0.011	0.047 ± 0.013	2.57 ± 0.82	9.91 ± 1.54 (3) *

Results represent means ± S.E.M., N=4, unless otherwise stated in parenthesis.

*Significantly different from the corresponding immersed condition (p<0.05).

Table 4.3. Effects of 6 days of aerial exposure on contents of free amino acids (FAAs), which showed significant changes, and total FAA (TFAA) in the liver and muscle of *Protopterus annectens*.

	Content (μmol g^{-1} wet mass)			
	Liver		Muscle	
FAA	Immersed	Terrestrial	Immersed	Terrestrial
Glutamate	3.36 ± 0.73	2.05 ± 0.45	0.18 ± 0.02	0.29 ± 0.03 *
Glutamine	n.d.	n.d.	0.26 ± 0.05	0.48 ± 0.05 *
Leucine	0.12 ± 0.01	0.12 ± 0.01	0.098 ± 0.033	0.21 ± 0.03 *
Proline	0.69 ± 0.11	0.25 ± 0.04 *	0.09 ± 0.01	0.099 ± 0.016
Threonine	0.204± 0.05	0.098 ± 0.015	0.26 ± 0.03	0.17 ± 0.01 *
TFAA	6.97 ± 1.15	4.61 ± 0.73	8.94 ± 1.62	8.75 ± 1.28

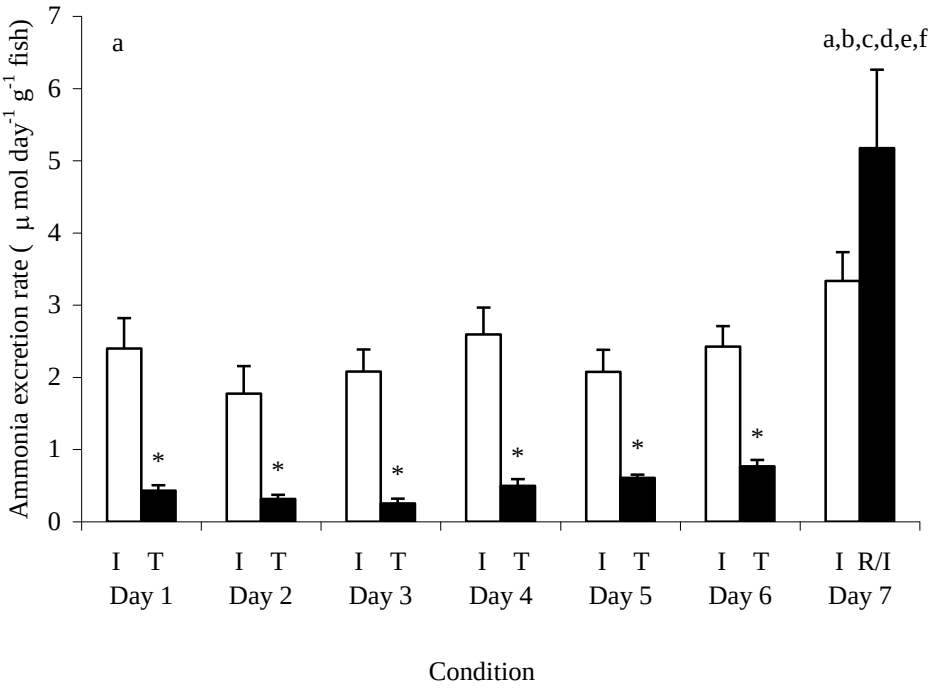
Values are means ± S.E.M. *N*=4.

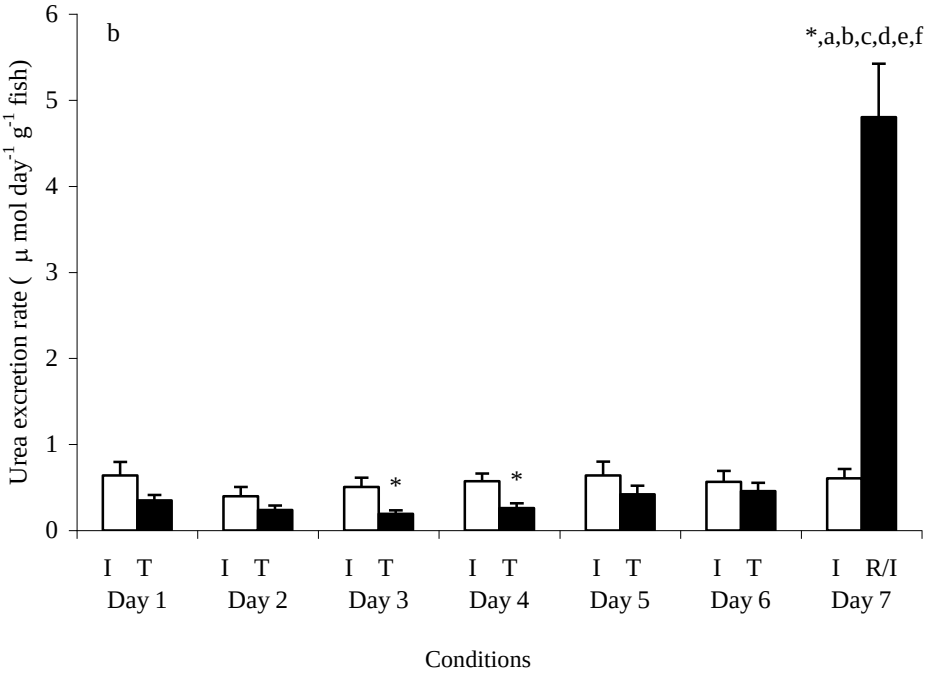
n.d.; not detectable (detection limit = 0.01 μmol g^{-1} wet mass)

*Significantly different from the corresponding immersed condition (p<0.05).

Fig. 4.1. Effects of 6 days of aerial exposure followed by 1 day of re-immersion in water on the rates of (a) ammonia excretion and (b) urea excretion in *Protopterus annectens*. Values are means ± S.E.M. I, immersed (N=4); T, terrestrial (N=8); R/I, re-immersed (N=4). *Significantly different from the corresponding immersed condition (p<0.05); [a]Significantly different from the corresponding day 1 condition (p<0.05); [b]Significantly different from the corresponding day 2 condition (p<0.05); [c]Significantly different from the corresponding day 3 condition (p<0.05); [d]Significantly different from the corresponding day 4 condition (p<0.05); [e]Significantly different from the corresponding day 5 condition (p<0.05); [f]Significantly different from the corresponding Day 6 condition (p<0.05).

Fig. 4.1.


a
a,b,c,d,e,f
Ammonia excretion rate (µ mol day-1 g-1 fish)
0
1
2
3
4
5
6
7
*
I T
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
I R/I
Day 7
Condition


b
*,a,b,c,d,e,f
Urea excretion rate (µ mol day-1 g-1 fish)
0
1
2
3
4
5
6
*
I T
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
I R/I
Day 7
Conditions

4.4. Discussion

4.4.1. *Presence of CPS III, not CPS I,* in P. annectens

It has been proposed that water-land transition in vertebrates involved the replacement of CPS III with CPS I in the mitochondria and the replacement of a mitochondrial arginase with a cytosolic enzyme in the liver (Mommsen and Walsh, 1989). Previous conception of lungfishes, like tetrapods, possessing CPS I led Mommsen and Walsh (1989) to conclude that the replacement of CPS III with CPS I occurred before the evolution of the extant lungfishes. However, our results revealed that the CPS from the liver of *P. annectens* had properties comparable to those of the marine stingray *Taeniura lymma* and different from those of the mouse *Mus musculus*. Thus, like *P. dolloi* (Chew et al., 2003b), *P. annectens* evidently possess CPS III instead of CPS I. Results obtained from this study, therefore, contradict and shed doubt on previous reports which claimed the presence of CPS I in *P. annectens* and *P. aethiopicus* (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989).

It is important to note that CPS III was discovered by Trammel and Campbell (1970) in invertebrates in late 1960s, and later verified to be present in fish by Anderson (1976) in mid 1970s. Before that, any CPS activity was assumed to be CPS I, and ammonia was used as the substrate in the assay medium as in the case of Janssens and Cohen (1966, 1968a). While CPS I would have low (or no) activity in the presence of glutamine, CPS III can utilize NH_4^+ as a substrate although the activity would be lower than that obtained with glutamine. So, the inclusion of NH_4^+ in the assay medium (Janssens and Cohen, 1966, 1968a) allowed for the detection of some CPS activities, which could be mistaken as CPS I, despite the fact that actually CPS III was present. On the other hand, Mommsen and Walsh (1989) did not present any numerical data on *P. aethiopicus* and *P. annectens* in their report, and therefore we were unable to compare what they regarded as CPS I activities with what we had obtained as CPS III activities in this study. More importantly, it is unclear about which method they (Mommsen and Walsh, 1989) had used to determine CPS activities from these lungfishes

because two different methods (colorimetric and radiometric) were cited in the same report. Thus, it becomes impossible to make a detailed methodological analysis of the reasons for the differences observed.

Mommsen and Walsh (1989, 1991) reported that GS was present exclusively in the cytosolic fractions of the liver of *P. aethiopicus* and *P. annectens*. However, contrary to those reports, substantial GS activities were detected in the liver mitochondria of *P. annectens* in this study. This is consistent with the presence of CPS III in the hepatic mitochondria of this African lungfish, because CPS III utilizes glutamine as a substrate. Therefore, despite the presence of cytosolic arginase in the livers of *P. annectens*, our results suggest that the evolution of CPS III to CPS I could not have occurred before the evolution of the extant lungfishes. In fact, our results are in support of the coelacanth/lungfish clade (Yokobori et al., 1994), and thus shed doubts on the current view on the evolution of CPS, and hence OUC, in vertebrates (Mommsen and Walsh, 1989, 1991).

4.4.2. Aerial exposure led to suppression in ammonia production in P. annectens

In terrestrial conditions, no water current is available to take away the excreted ammonia from the gills; and branchial ammonia excretion by diffusion is repressed. Indeed, ammonia excretion rates in *P. annectens* exposed to terrestrial conditions decreased significantly. This should theoretically result in accumulation of ammonia in their tissues. However, there were no changes in the ammonia contents in the muscle, liver, plasma and brain of *P. annectens* after 6 days of aerial exposure. Like *P. dolloi* (Chew et al., 2003b), *P. annectens* detoxified ammonia to urea during aerial exposure, and the excess urea was mainly stored in the body. However, unlike *P. dolloi*, there were only relatively small increases in rates of urea synthesis in *P. annectens* during 6 days of aerial exposure (see below).

For *P. annectens*, the decrease in ammonia excretion in a 100 g specimen during the 6-days period amounted to [(2.4-0.4) + (1.8-0.3) + (2-0.25) + (2.6-0.5) + (2-0.6) + (2.4-0.8)]

μmol g^{-1} x 100 g = 1035 μmol (from Fig 4.1a). The excess amount of urea accumulated in the body of (from Table 4.2), minus the decrease in urea excretion during the 6 days in (from Fig. 4.2b), a 100 g specimen can be calculated as [(8.23-3.3) μmol g^{-1} x 55g + (13.1-4.79) μmol g^{-1} x 2 g + (11.3 -4.01) mmol l^{-1} x 1 ml + (9.91-2.57) μmol g^{-1} x 0.3 g] – [(0.3 + 0.2 + 0.3 + 0.3 + 0.2 + 0.15) μmol g^{-1} x 100 g], or 152.3 μmol. This amount of urea is equivalent to 152.3 x 2 or 304.5 μmol of ammonia. Again, the deficit (1035-304.5) of 730.5 μmol indicates the occurrence of a reduction in the rate of endogenous ammonia production in *P. annectens* during aerial exposure. The deficit of 730.5 μmol N corresponds to a reduction of 1.22 μmol day^{-1} g^{-1} in ammonia production, which is equivalent to 37% (=1.22×100/3.3) of the rate of ammonia + urea production (3.3 μmol day^{-1} g^{-1}) in the control fish kept in water.

It is important to note that the change in ammonia excretion during recovery in water on day 7 for *P. annectens* was different from that for *P. dolloi* (Chew et al., 2003b). The suppression of ammonia production in *P. dolloi* continued into day 7 because the ammonia excretion rate remained low (12% of the control) during recovery in water (Chew et al., 2003b). By contrast, the rate of ammonia excretion in *P. annectens* during recovery on day 7 was comparable to the control level. So, while *P. annectens* could suppress ammonia production during aerial exposure, they could restore normal nitrogen metabolic rate within 24 h.

Chew et al. (2003b) observed a significant decrease in the TFAA content in the liver of *P. dolloi* after 6 days of aerial exposure, which suggest that a suppression of ammonia production through reductions in protein degradation and amino acid catabolism would have occurred. By contrast, there were no significant changes in TFAA contents, in spite of changes in contents of several FAAs including some essential amino acids, in the muscles and liver of *P. annectens* exposed to terrestrial conditions for 6 day. If indeed reductions in ammonia production were achieved through reductions in catabolism of amino acids, these results imply that comparable reductions in protein degradation and amino acid catabolism

would have occurred. Only then, the steady state levels of many of the FAAs were unaffected, resulting in the relatively unchanged TFAA contents.

4.4.3. Aerial exposure led to increases in rates of urea synthesis in P. annectens

The rate of urea synthesis in a submerged *P. annectens* was approximately 0.55 µmol d^{-1} g^{-1} (averaged of the control rates during 6 days; Fig. 4.1b). The amount of urea synthesized in a 100 g specimen during 6 days of aerial exposure is equal to [(0.3 + 0.2 + 0.2 + 0.3 + 0.4 + 0.45) µmol g^{-1} x 100 g] + [(8.23-3.3) µmol g^{-1} x 55g + (13.1-4.79) µmol g^{-1} x 2 g + (11.3 -4.01) mmol l^{-1} x 1 ml + (9.91-2.57) µmol g^{-1} x 0.3 g] or 487.3 µmol. This is equivalent to a rate of 487.3 µmol/(100 g x 6 days) or 0.81 µmol day^{-1} g^{-1}. Thus, the rate of urea synthesis was up-regulated (0.81/0.55) or 1.47-fold to detoxify the endogenous ammonia, which could not be excreted as NH_3 during aerial exposure.

Chew et al., (2003b) reported that the rate of urea synthesis (8.6-fold) and the activities of OUC enzymes, including CPS III (3.8-fold), in *P. dolloi* were enhanced by 6 days of aerial exposure. However, *P. annectens* apparently depended more on reductions in ammonia production (see above) than on increases in OUC capacities to ameliorate ammonia toxicity. This was evidently reflected by the lack of significant increases in CPS III activities in the livers of *P. annectens* exposed to air for 6 days, despite significant increases in activities of GS (2.9-fold) and OTC (1.4-fold) in *P. annectens*. There can be two major factors leading to these observations. Firstly, the normal ammonia excretion rates of *P. annectens* (2.37±0.41 µmol day^{-1} g^{-1}) was lower than that of *P. dolloi* (6.25±0.82 µmol day^{-1} g^{-1}, Chew et al., 2003b); hence, the addition load of ammonia to be detoxified to urea was smaller in *P. annectens*. Secondly, *P. annectens* could achieve a relatively greater degree of suppression of ammonia production (37%) than *P. dolloi* (28%; Chew et al., 2003b), which would help to reduce the load further. As a result, the slightly increased urea synthesis rate could be handled adequately by the normal capacity of CPS. However, the reasons behind

the changes in GS and OTC activities in *P. annectens* during aerial exposure remain unclear at present.

A major portion of the urea synthesized in *P. annectens* during aerial exposure was stored in their tissues and organs. Upon re-immersion on day 7, there was a significant increase (10-fold greater than the control values) in its urea excretion rates to remove the stored urea, in spite of ammonia excretion rates being comparable with the submerged controls.

4.4.4. A comparative perspective

Contrary to previous reports, we demonstrated the presence of CPS III in *P. annectens*. Since *P. dolloi* also possesses CPS III (Chew et al., 2003b) it can be concluded that the evolution of CPS III to CPS I is likely to have occurred after and not before the evolution of the extant lungfishes. *P. annectens* did not up-regulate CPS III activities after 6 days of aerial exposure; hence, it responded differently from *P. dolloi*, the CPS III of which increases 3.8-fold after being exposed to similar experimental conditions (Chew et al., 2003b). Chew et al. (2004) reported that the rate of urea synthesis and the activities of CPS III in *P. dolloi* increased significantly after 40 days of aestivation. However, results from this study indicate that *P. annectens* depended more on a reduction in ammonia production than an increase in urea synthesis to defend against ammonia toxicity during 6 days of aerial exposure. More importantly, our results suggest the presence of subtle differences in responses to aerial exposure with respect to nitrogen metabolism and excretion in various African lungfishes.

4.5. Summary

The objectives of this study were (1) to determine the type of CPS present, and the compartmentalization of arginase, in the liver of the African lungfish, *Protopterus annectens*, and (2) to elucidate if *P. annectens* were capable of increasing the rates of urea synthesis and capacities of the OUC during 6 days of aerial exposure without undergoing aestivation. Like another African lungfish *Protopterus dolloi* reported elsewhere, the CPS activities from the liver of *P. annectens* had properties similar to those of marine elasmobranchs, but dissimilar to those of mammals. Hence, they possessed CPS III, and not CPS I as reported previously. CPS III was present exclusively in the liver mitochondria of *P. annectens*, but the majority of the arginase activities were present in the cytosolic fractions of the liver. GS activity was also detected in the hepatic mitochondria of *P. annectens*. Therefore, results from this study suggest that the evolution of CPS III to CPS I is unlikely to have occurred before the evolution of extant lungfishes as suggested previously, and shed doubt on the current view on the evolution of CPS and OUC in vertebrates. Aerial exposure led to a significant decrease in the rate of ammonia excretion in *P. annectens*, but there was no accumulation of ammonia in their tissues. However, urea contents in their tissues increased significantly after 6 days of aerial exposure. The rates of urea synthesis in *P. annectens* increased 1.47-fold, which was smaller than that of *P. dolloi* (8.6-fold) reported elsewhere. In addition, unlike *P. dolloi*, 6 days of aerial exposure had no significant effect on the hepatic CPS III activities of *P. annectens*. By contrast, aerial exposure induced relatively greater degrees of reductions in ammonia production in *P. annectens* (37%) than *P. dolloi* (27.5%) as previously reported. Thus, these results suggest that various species of African lungfishes respond to aerial exposure differently with respect to nitrogen metabolism and excretion, and it can be concluded that *P. annectens* depended more on reductions in ammonia production than on increases in urea synthesis, as in the case of *P. dolloi*, to ameliorate ammonia toxicity when exposed to terrestrial conditions.

5. Chapter 2:

Increased urea synthesis and/or suppressed ammonia production in the African lungfish, *Protopterus annectens*, during aestivation in air or mud

5.1. Introduction

Lungfishes, characterized by the possession of a lung opening off the ventral side of the oesophagus, are an archaic group of fishes that belongs to the class Sarcopterygii. There are six species of extant lungfishes worldwide, of which four (*Protopterus aethiopicus*, *Protopterus annectens*, *Protopterus amphibicus* and *Protopterus dolloi*) can be found in Africa (Greenwood, 1987). African lungfishes depend completely on aerial respiration (Graham, 1997) and are able to survive extended periods of drought through aestivation. They are ammonotelic in water, producing ammonia and excreting it as NH_3 by diffusion across the branchial and cutaneous epithelia (Graham, 1997). However, when stranded on land during a drought, ammonia excretion would be impeded due to a lack of water to flush the branchial and cutaneous surfaces, and there would be a build-up of ammonia in the body. Ammonia is toxic (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip et al., 2001b; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002), and acts on the central nervous system of vertebrates, including fish, causing hyperventilation (Hillaby and Randall, 1979; McKenzie et al., 1993), hyperexcitability, convulsions, coma, and finally death. Therefore, African lungfishes have to defend against ammonia toxicity when out of water.

Many air-breathing tropical fishes have evolved mechanisms to defend against ammonia toxicity during aerial exposure (Ip et al., 2001b, 2004a, b; Chew et al. 2006). These mechanisms include the active excretion of NH_4^+ (Randall et al., 1999, 2004; Ip et al., 2004c, 2004d; Tay et al., 2006; Chew et al., 2007), the conversion of ammonia to less toxic compounds such as glutamine (Jow et al., 1999; Ip et al., 2001a; Tay et al., 2003) or urea (Smith, 1930; Janssens and Cohen, 1968b; Chew et al., 2003b, 2004; Loong et al., 2005 Ip et al., 2005f), the suppression of ammonia production in general through a reduction in amino acid catabolism (Jow et al., 1999; Lim et al., 2001, Ip et al., 2001a; Chew et al., 2001, 2003b, 2004; Tay et al., 2003; Walsh et al., 2004; Loong et al., 2005; Ip et al., 2005c, d, f), the reduction in ammonia production via partial amino acid catabolism leading to the formation

of alanine (Ip et al., 2001c; Chew et al., 2003c), and the excretion of NH_3 into air by volatilisation (Tsui et al., 2002; Frick and Wright, 2002).

The two major mechanisms adopted by African lungfishes to deal with ammonia toxicity during emersion or aestivation are increase in urea synthesis and decrease in ammonia production (Smith, 1930, 1935; Janssens, 1964; Janssens and Cohen, 1968a, b; Chew et al., 2003b, 2004; Loong et al., 2005; Ip et al., 2005f). African lungfishes are ureogenic and possess a full complement of hepatic ornithine-urea cycle (OUC) enzymes (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989) that include carbamoyl phosphate synthetase III (CPS III) (Chew et al., 2003b; Loong et al., 2005). Because of this, the main focus in the past was on the involvement of increased urea synthesis in the defence against ammonia toxicity (Smith, 1930, 1935; Janssens, 1964; Janssens and Cohen, 1968a, b), but the importance of decreased ammonia production was largely neglected until recently (Chew et al., 2003b, 2004; Loong et al., 2005; Ip et al., 2005f). Chew et al. (2004) demonstrated that *P. dolloi* detoxified endogenous ammonia to urea, and the rate of urea synthesis increased 2.4- and 3.8-fold during 6 and 40 days (equivalent to 12 and 46 days, respectively, in this study), respectively, of aestivation in air. In addition, during the first 6 days and the following 34 days of aestivation, the rate of ammonia production in *P. dolloi* decreased to 26% and 28%, respectively, of the day 0 control value. Based on the method of Chew et al. (2004), the author succeeded in inducing and perpetuating aestivation in *P. annectens* in dried mucus cocoons in air. In addition, the author could induce *P. annectens* to aestivate inside soft mud. Thus, this offered the author the unique opportunity to evaluate the importance of increased urea synthesis and decreased ammonia production in fish aestivating in mud versus those aestivating in air.

Urea synthesis in fish is energy intensive as a total of 5 mol of ATP are hydrolysed for each mole of urea synthesised, which corresponds to 2.5 mol of ATP used for each mole of nitrogen assimilated. Hence, increased urea synthesis may not be an effective strategy for

fish aestivating in hypoxic mud because environmental hypoxia prescribes a low efficiency of ATP production through anaerobic pathways (Hochachka, 1980). Indeed, it has been reported recently that much less glutamine, the formation of which is also ATP-dependent, accumulates in tissues of the non-ureogenic swamp eel, *Monopterus albus*, kept in mud for 40 days as compared with fish kept in air for a similar period (Chew et al. 2005a). Therefore, the first objective of this study was to investigate whether aestivation for 12 or 46 days in air or in mud would lead to accumulations of ammonia and/or urea in the body of *P. annectens*. The second objective was to examine whether fish would adopt increased urea synthesis as a strategy to detoxify endogenous ammonia during aestivation in air or in mud. To achieve that, the author determined tissue urea contents in, and activities of enzymes associated with urea synthesis from, the liver of *P. annectens* after 12 or 46 days of aestivation in air or in mud. The hypothesis tested was that fish aestivating in mud, unlike those aestivating in air, would not adopt this strategy to ameliorate ammonia toxicity in order to conserve cellular energy under a presumably hypoxic environment. The author hypothesized that a reduction in ammonia production could be a more effective strategy to deal with ammonia toxicity in fish aestivating in mud. Thus, the third objective was to elucidate through calculations whether the degrees of reduction in ammonia production in fish aestivated in mud for 12 or 46 days were greater than those in fish aestivated in air for similar periods.

5.2. Materials and methods

5.2.1. Animals

Specimens of *P. annectens* weighing 75-155 g body mass were imported from Africa through a local fish farm in Singapore. Fish were maintained in plastic aquaria filled with water containing 2.3 mmol l^{-1} Na^{+}, 0.54 mmol l^{-1} K^{+}, 0.95 mmol l^{-1} Ca^{++}, 0.08 mmol l^{-1} Mg^{++}, 3.4 mmol l^{-1} Cl^{-} and 0.6 mmol l^{-1} M HCO_3^{-}, at pH 7.0 and at 25^{o}C in the laboratory and water was changed daily. No attempt was made to separate the sexes. Fishes were acclimated to laboratory conditions for at least one month. During the acclimatization period, they were fed frozen bloodworms. Food was withdrawn 96 hour before experiments, which allowed sufficient time for the gut to be emptied of all food and waste. The wet mass of the fish before and after exposure to environmental conditions was determined to the nearest 0.1 g using a Shimadzu (Shimadzu Co., Kyoto, Japan) animal balance.

5.2.2. Exposure of fish to experimental conditions and collection of samples

In this study, fish kept individually in 2 l of dechlorinated tap water in plastic tanks (L20.5 cm x W14.5 cm x H6 cm), without food supply for 12 or 46 days served as fasting controls. Water was changed daily, and water samples (3 ml) were collected 24 h before the change of water for the determination of ammonia and urea excretion rates. Ammonia and urea were determined according to the methods of Jow et al. (1999). Results were expressed as averaged ammonia and urea excretion rates (μmol day^{-1} g^{-1} fish) over a 6-day period, except for the last 4 days.

On days 0 (day 0 control), 12 and 46 (fasting controls) fish were killed with a strong blow to the head. Collection of blood through cannulation could not be adopted in this study because the wound and tubing would interfere with the aestivation process, especially during aestivation in mud. Therefore, blood was collected through caudal puncture into a heparinized (sodium heparin) syringe. The collected blood was centrifuged at 4,000 x*g* at 4^{o}C

for 10 min to obtain the plasma. The plasma was deproteinized in an equal volume (v/v) of ice-cold 6% trichloroacetic acid (TCA) and centrifuged at 10,000 x*g* at 4^{o}C for 15 min. The resulting supernatant was kept at -80^{o}C until analysis. The lateral muscle, liver, gut and brain were quickly excised and freeze-clamped with aluminium tongs pre-cooled in liquid nitrogen. Frozen samples were kept at -80^{o}C until analysis.

It has been reported recently that increases in salinity and changes in ionic composition in the external medium could induce a decrease in ammonia production, which is an important facet of aestivation, in *P. dolloi* (Ip et al., 2005c). So, following the procedure of Chew et al. (2004) for *P. dolloi*, *P. annectens* were induced to aestivate at 27-29^{o}C and 85-90% humidity individually in plastic tanks (L29 cm x W19 cm x H17.5 cm) containing 15 ml dechlorinated tap water (made 0.3‰ with seawater). It took approximately 6 days for the fish to be encased in a brown dried mucus cocoon. In this study, these 6 days were counted as part of the aestivation period. So, for a fish aestivated for 12 days, it would have spent at least 6 days within the dried mucus cocoon. Similarly, a fish aestivated for 46 days would have been in the cocoon for approximately 40 days. Fish were killed on days 12 or 46, and tissues sampled as described above.

Dried mud collected from the bottom of freshwater ponds was purchased from Hua Hing Trading Co. (Singapore). The dried mud was soaked in dechlorinated tap water for at least 2 days, and mixed into a thick paste (approximately 30% water content) by hand. Artificial muddy substrata (19 kg dry mass) with a minimum depth of 15 cm were made in plastic tanks (L29 cm x W19 cm x H17.5 cm). Fish (one per tank) were allowed to bury at liberty into mud, which took 2-12 h. A small amount of water (approximately 100 ml) was evenly spread on to the surface of the mud every 4-5 days to prevent the surface mud from drying up and cracking. Attempts were made to monitor the pO_2 in the mud using an Ocean Optics fibre optic O_2 sensing system with a fibre optic O_2 sensor (FOXY AL300), but the pO_2 in the mud was below the detection limit. Fish were killed on days 12 or 46, and tissues

sampled as described above. When aestivating fish were recovered from the mud on days 12 and 46, it was discovered that they burrowed to a depth of 10-15 cm, and in all cases they were encased in a layer of dried mucus, meaning that they were not in direct contact with mud. Therefore, it is highly unlikely that they could have excreted significant amount of wastes into the surrounding substratum. In addition, there was a small air passage which connected the point of entry from the mud surface to the aestivating fish. However, in all cases, the anterior end of the fish was observed to be orientated away from the air passage.

5.2.3. Determination of ammonia, urea and free amino acids (FAAs)

The frozen samples were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% TCA at 24,000 revs min^{-1} for 20 sec each using an Ultra-Turrax homogenizer with intervals of 10 sec between each homogenization. The homogenate was centrifuged at 10,000 x*g* at 4^{o}C for 15 min, and the supernatant obtained was kept at -80^{o}C until analysis.

For ammonia, urea and FAA analysis, the methods were similar to those presented in Chapter 1, section 4.2.3. The total FAA (TFAA) concentration was calculated by the summation of all FAAs, while total essential FAA (TEFAA) concentration was calculated as the sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine concentrations. The amount of urea accumulated in a hypothetical 100 g fish, which contained 55 g muscle, 2 g liver, 0.3 g brain and 1 ml plasma, was calculated according to method of Loong et al. (2005).

5.2.4. Determination of activities of hepatic OUC enzymes

Frozen liver samples were homogenized in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} Hepes (pH 7.6), 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol and 0.5 mmol l^{-1} PMSF. The homogenate was sonicated (110 W, 20 kHz;

Misonix Incorporated Farmingdale, NY, USA) three times for 20 s each, with a 10 s break between each sonication. The sonicated sample was centrifuged at 10,000 x*g* at 4°C for 15 min. After centrifugation, the supernatant was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with the extraction buffer without EDTA and PMSF. The filtrate obtained was used directly for enzymes assay as described in Chapter 1, section 4.2.2. Protein assay (Bradford, 1976) was performed on the sample before and after passing through the column to estimate the dilution factor involved.

5.2.5. Determination of blood pO_2 and muscle ATP content

After a strong blow to the head, blood of fish fasted for 46 days in water or aestivated in air or mud for 46 days were collected through caudal puncture, and blood pO_2 was determined immediately using a Medica Easy blood gas analyzer (Medica Corporation, Bedford, MA, USA), which has been calibrated to determine pO_2 in *P. dolloi* blood at 25^{o}C using the equipment and methods as described in Perry et al. (2005a, b).

The frozen muscle The frozen muscle samples were weighed, ground to powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% perchloric acid at 24,000 revs min^{-1} for 20 sec each using an Ultra-Turrax homogenizer with intervals of 10 sec between each homogenization. The homogenate was centrifuged at 10,000 x*g* at 4°C for 15 min. The supernatant obtained was neutralized with 5 mol l^{-1} $KHCO_3$ and centrifuged at 10,000*g* to remove the potassium perchlorate precipitate. The resultant supernatant was used for the determination of ATP using the method of Scheibel et al. (1968). Results were expressed in µmol g^{-1} muscle.

5.2.6. Statistical analyses

Results were presented as means ± standard errors of the mean (S.E.M.). One-way analysis of variance followed by Student-Neuman-Keul's multiple range test and Students-t

test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $p<0.05$.

5.3. Results

5.3.1. Effects of 12 or 46 days of fasting (control fishes)

The ammonia and urea excretion rates (*N*=5) of *P. annectens* on day 0 (fasted for 96 hour) in water were 2.47 ± 0.48 and 0.78 ± 0.17 μmol day^{-1} g^{-1}, respectively. The steady state level of urea in the body is maintained by the rate of urea excretion being balanced by the rate of urea production. In the case of *P. annectens*, urea is mainly produced through urea synthesis in the liver. Hence, the rate of urea synthesis in a control *P. annectens* on day 0 can be estimated as 0.78 μmol day^{-1} g^{-1} fish (Table 5.1). In addition, the rate of ammonia production in the day 0 fish can be estimated as the summation of the rates of ammonia-N and urea-N excretion (one mole of urea contains two moles of N), i.e., 2.47 + (0.78 x 2) or 4.0 μmol day^{-1} g^{-1} (Table 5.1). During the subsequent 46 days of fasting, there was a progressive increase in the amount of total nitrogenous waste excreted, which was expressed as significant increases in the rate of ammonia excretion between day 31 and day 46 and the rate of urea excretion between day 19 and day 46 (Fig. 5.1).

The ammonia contents (*N*=5) in the muscle, liver, brain and plasma of *P. annectens* on day 0 (fasted for 96 h) were 0.52 ± 0.27 μmol g^{-1}, 1.23 ± 0.38 μmol g^{-1}, 0.89 ± 0.23 μmol g^{-1} and 0.29 ± 0.09 μmol ml^{-1}, respectively. The respective urea contents (*N*=5) were 3.32 ± 0.98 μmol g^{-1}, 4.53 ± 1.21 μmol g^{-1}, 2.89 ± 0.93 μmol g^{-1} and 4.23 ± 1.29 μmol ml^{-1}. Fasting for 12 days in freshwater had no significant effects on the ammonia and urea contents in various tissues of *P. annectens* (Fig. 5.2) as compared with the day 0 control. Although 46 days of fasting did not affect the tissue ammonia contents, it led to a slight but insignificant increase in urea contents in the muscle, liver, brain and plasma of *P. annectens* (Fig. 5.3b). The brain ammonia content presented herein is higher than that reported by Loong et al. (2005) because of a reporting error in that report.

The activities of GS and OUC enzymes from the liver of *P. annectens* fasted in water for 12 or 46 days were comparable (Table 5.2).

5.3.2. Effects of 12 or 46 days of aestivation in air

There were no significant increases in ammonia contents in the muscle, liver, brain and plasma of *P. annectens* after 12 days of aestivation in air compared with the fasted control (Fig. 5.2a). Surprisingly, the ammonia level in the liver of specimens aestivated for 46 days in air decreased significantly to 30% of the value of control fish fasted for a similar period in water (Fig. 5.3a).

Urea contents in the muscle, liver, brain and plasma of fish aestivated for 12 days in air were 16-, 11-, 13- and 11-fold, respectively, greater than the corresponding values of fish fasted for 12 days (Fig. 5.2b). From Fig. 5.2b, the excess amount of urea accumulated in the body of a 100 g fish after 12 days of aestivation in air can be calculated to be 2483 μmol (or 4966 μmol N) 100 g^{-1} fish (Table 5.1). After 46 days of aestivation in air, the urea contents in the muscle, liver, brain and plasma were 15-, 16-, 16- and 16-fold, respectively, greater than the corresponding values of the control fasted for 46 days (Fig. 5.3b). Furthermore, urea levels in the muscle, liver, brain and plasma of fish aestivated for 46 days in air were 2.1-, 2.7-, 2.2- and 2.7-fold, respectively, greater than the corresponding values of fish aestivated for 12 days in air. From Fig. 5.3b, it can be calculated that 5188 μmol (or 10376 μmol N) of excess urea would have accumulated in a 100 g fish (Table 5.1).

Aestivation for 12 or 46 days in air had no significant effects on the activities of GS and OUC enzymes from the liver of *P. annectens* compared with fish fasted for similar periods (Table 5.2).

There was a significant increase in β-alanine content in the muscle of fish aestivated for 12 days in air, but there was no change in the muscle TFAA content compared with the

fasted control (Table 5.3). As for the liver, 12 days of aestivation in air led to a significant increase in the arginine, glycine and histidine contents and significant decreases in contents of glutamate and glutamine, but the TFAA content remained unchanged (Table 5.3). In contrast, contents of brain glutamate, glutamine, glycine, proline, serine, taurine, threonine, tyrosine, valine and TFAA increased significantly in fish aestivated for 12 days in air (Table 5.3). After 46 days of aestivation in air, there was no change in the TFAA content in the muscle of *P. annectens*. However, 46 days of aestivation in air led to significant decreases in contents of alanine, glutamate, glutamine, lysine and serine in the liver, which led to a significant decrease in the TFAA content (Table 5.4). After 46 days of aestivation in air, there were significant decreases in contents of arginine, alanine and serine in the brain (Table 5.4). However, unlike fish aestivated for 12 days in air, there were no significant changes in contents of glutamate, glutamine and TFAA in the brain of fish aestivated for 46 days in air.

The masses of 4 individual fish at the start of the experiment were 113, 77, 99 and 123 g, which decreased to 109, 73, 93 and 118 g, respectively, after 12 days of aestivation in air; the average decrease was 4.73%. For fish aestivating for 46 days in air, the wet masses of 4 individual fish were 92, 155, 105 and 102 g before experiment, and they decreased to 72, 130, 89 and 84 g, respectively after aestivation. There was an average decrease of 17.7% in the mass of these fish. The pO_2 level in the blood of fish aestivated in air for 46 days (50.5 ± 13.0 mm Hg; *N*=4) was not significantly different from that of fish kept in water (43.8 ± 9.4 mm Hg; *N*=5). In addition, the muscle ATP content (*N*=4) of fish aestivated in air for 46 days (8.78 ± 1.65 μmol g^{-1}) was comparable to the control value for fish kept in water (7.65 ± 1.53 μmol g^{-1}).

5.3.3. Effects of 12 or 46 days of aestivation in mud

Similar to fish aestivating in air, there was no significant increase in ammonia contents in the muscle, liver, brain and plasma of *P. annectens* after 12 days of aestivation in

mud compared with the fasted control (Fig. 5.2a). Also, ammonia levels in the liver of fish aestivated for 46 days in mud decreased significantly to 20% of the control value of fish fasted for a similar period in water (Fig. 5.3a).

Surprisingly, urea contents in all four tissues were comparable between fish aestivated for 12 and those aestivated for 46 days in mud. Urea contents in the muscle, liver, brain and plasma of fish aestivated for 12 days in mud, despite apparent increases of 3.1-, 3.2-, 3- and 3.1-fold, respectively, were not significantly different from the control fasted for the same period (Fig. 5.2b). Taking these insignificant increases in urea contents into consideration, the amount of urea accumulated in a 100 g fish after 12 days of aestivation in mud was 366 µmol (or 732 µmol N) (Table 5.1). In contrast, 46 days of aestivation in mud had no significant effects on urea contents in the muscle, liver, brain and plasma (Fig. 5.3b); and, the minute amount of excess urea accumulated in a 100 g fish during 46 days of aestivation is calculated to be 111 µmol (or 222 µmol N; Table 5.1).

Aestivation for 12 or 46 days in mud had no significant effects on the activities of GS and OUC enzymes from the liver of *P. annectens* compared with the corresponding fasted control (Table 5.2).

The content of TFAA remained unchanged in the muscle of *P. annectens* aestivated for 12 days (Table 5.3) in mud as compared with the control fasted for a similar period in water. Unlike fish aestivating in air, 12 days of aestivation in mud had no significant effect on the content of arginine in the liver (Table 5.3). Both liver glutamate and glutamine contents decreased significantly in fish aestivated for 12 days in mud, but there was no change in the liver TFAA content (Table 5.3). The arginine, alanine and glutamate contents in the brain were significantly lower in fish aestivated for 12 days in mud compared with the fasted control (Table 5.3). On the other hand, the glutamine content remained unchanged in the brain of fish aestivated for 12 days in mud (Table 5.3). Overall, the TFAA content in the brain of fish aestivated in mud for 12 days decreased significantly (Table 5.3). Forty-six days

of aestivation in mud had no significant effect on the TFAA content in the muscle of *P. annectens*, but there were significant decreases in contents of alanine, arginine, lysine and serine in the liver of these experimental fish (Table 5.4). In addition, the liver glutamate, glutamine and TFAA contents were significantly lower in fish aestivated for 46 days in mud as compared to those of the fasted control (Table 5.4). After 46 days of aestivation in mud, there was a significant decrease in contents of arginine and serine, but no significant changes in glutamate, glutamine and TFAA contents, in the brain of *P. annectens* (Table 5.4).

The masses of 4 individual fish at the start of the experiment were 80, 88, 96 and 108 g, and they remained unchanged (83, 90, 100 and 107 g, respectively) after 12 days of aestivation in mud. Similarly, the wet masses of 4 individual fish before aestivation were 103, 126, 60 and 133 g, which remained unchanged (101, 128, 62 and 134 g, respectively) after 46 days of aestivation in mud. The pO_2 level in the blood of fish aestivated in mud for 46 days (28.8 ± 6.3 mm Hg; *N*=4) was significantly lower than that of fish kept in water (43.8 ± 9.4 mm Hg; *N*=5). In addition, the muscle ATP content (*N*=4) of fish aestivated in mud for 46 days (3.84 ± 1.09 μmol g^{-1}) was significantly lower than that of the control fish kept in water (7.65 ± 1.53 μmol g^{-1}).

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Table 5.1. A summary of the estimated deficit in nitrogenous excretion (μmol N), the estimated amount of urea-N accumulated (μmol N), and estimated rates of urea synthesis (μmol urea day^{-1} g^{-1} fish) and ammonia production (μmol N day^{-1} g^{-1} fish) in a hypothetical 100 g *Protopterus annectens* aestivated in air or mud for 12 or 46 days in comparison with the estimated rate of urea synthesis and ammonia production in the control fish kept in water on day 0.

P. annectens which weighed	In water	In air		In mud	
100 g	Day 0	12 days	46 days	12 days	46 days
Deficit in N-excretion	--	4790	34028	4790	34028
Excess urea-N accumulated	--	4966	10376	732	222
Rate of urea synthesis	0.78	2.07	1.1	0.31	0.02
Rate of ammonia production	4.0	4.1	2.26	0.61	0.048

Table 5.2. Activities (μmol min^{-1} g^{-1} wet mass) of glutamine synthetase (GS), carbamoyl phosphate synthetase (CPS III), ornithine transcarbamoylase (OTC), argininosuccinate synthetase + lyase (ASS+ L) and arginase from the liver of *Protopterus annectens* kept in freshwater (control), aestivated in air, or aestivated in mud for 12 or 46 days as compared with control fish fasted for 12 or 46 days in freshwater.

Enzymes	Enzyme activity (μmol min^{-1} g^{-1} wet mass)					
	12 days in freshwater (Control)	12 days aestivation in air	12 days aestivation in mud	46 days in freshwater (Control)	46 days aestivation in air	46 days aestivation in mud
GS	0.71 ± 0.14	2.5 ± 1.0	0.92 ± 0.22	0.72 ± 0.12	0.74 ± 0.26	0.85 ± 0.14
CPS III	0.12 ± 0.03	0.17 ± 0.03	0.16 ± 0.06	0.13 ± 0.04	0.32 ± 0.13	0.22 ± 0.08
OTC	37 ± 7	28 ± 3	29 ± 7	29 ± 7	49 ± 13	25 ± 7
ASS + L	0.69 ± 0.12	0.72 ± 0.12	0.29 ± 0.08	0.63 ± 0.19	0.81 ± 0.18	0.37 ± 0.11
Arginase	239 ± 51	352 ± 89	171 ± 24	302 ± 78	319 ± 69	194 ± 21

Results represent means ± SEM (*N*=5).

Table 5.3. Contents (μmol g^{-1} tissue) of various free amino acids (FAAs), which showed significant changes, and total FAA (TFAA) in the muscle, liver and brain of *Protopterus annectens* fasted in freshwater (control), aestivated in air, or aestivated in mud for 12 days

Tissue	FAA	Content (μmol g^{-1} tissue)		
		12 days in freshwater (control)	12 days aestivation (in air)	12 days aestivation (in mud)
Muscle	β-alanine	0.016 ± 0.001	0.064 ± 0.015[a]	0.035 ± 0.005
	Lysine	0.84 ± 0.08	0.62 ± 0.05	0.99 ± 0.11[b]
	TFAA	2.92 ± 0.24	4.82 ± 1.47	3.95 ± 0.37
Liver	Arginine	0.19 ± 0.02	0.39 ± 0.08[a]	0.11 ± 0.03[b]
	Glutamate	2.51 ± 0.42	1.31 ± 0.20[a]	1.16 ± 0.33[a]
	Glutamine	0.31 ± 0.09	0.045 ± 0.014[a]	0.085 ± 0.044[a]
	Glycine	0.24 ± 0.02	0.76 ± 0.12[a]	0.30 ± 0.09[b]
	Histidine	0.044 ± 0.004	0.091 ± 0.009[a]	0.090 ± 0.017[a]
	Phenylalanine	0.029 ± 0.003	0.016 ± 0.001	0.036 ± 0.006[b]
	TFAA	6.65 ± 0.81	5.74 ± 0.47	4.97 ± 0.91
Brain	Alanine	0.15 ± 0.01	0.18 ±0.02	0.079 ± 0.010[ab]
	Arginine	0.15 ± 0.01	0.13 ± 0.01	0.077 ± 0.001[ab]
	Aspartate	0.58 ± 0.04	0.93 ± 0.16[a]	0.31 ± 0.03[b]
	β-alanine	0.032 ± 0.003	0.055 ± 0.008[a]	0.025 ± 0.003[b]
	Glutamate	4.57 ± 0.18	6.85 ± 0.37[a]	3.22 ± 0.26[ab]
	Glutamine	2.71 ± 0.11	5.80 ± 0.33[a]	2.07 ± 0.20[b]
	Glycine	0.27 ± 0.01	0.57 ± 0.02[a]	0.40 ± 0.05[ab]
	Histidine	0.046 ± 0.002	0.052 ± 0.004	0.038 ± 0.004[b]
	Lysine	0.93 ± 0.05	1.03 ± 0.08	0.76 ± 0.04[b]
	Proline	0.056 ± 0.004	0.085 ± 0.011[a]	0.043 ± 0.009[b]
	Serine	0.41 ± 0.04	0.85 ± 0.09[a]	0.32 ± 0.04[b]
	Taurine	0.19 ± 0.01	0.41 ± 0.04[a]	0.29 ± 0.01[ab]
	Threonine	0.20 ± 0.02	0.47 ± 0.01[a]	0.18 ± 0.02[b]
	Tryptophan	0.15 ± 0.05	0.16 ± 0.06	0.041 ± 0.041
	Tyrosine	0.062 ± 0.003	0.13 ± 0.02[a]	0.083 ± 0.019[b]
	Valine	0.058 ± 0.007	0.11 ± 0.01[a]	0.091 ± 0.006[a]
	TFAA	10.8 ± 0.2	18.1 ± 0.9[a]	8.28 ± 0.41[ab]

Values are means ± SEM (N=4).

[a] Significantly different from the corresponding control condition (p<0.05).

[b] Significantly different from the corresponding aestivation condition (p<0.05).

Table 5.4. Contents (μmol g^{-1} tissue) of various free amino acids (FAAs), which showed significant changes, and total FAA (TFAA) in the muscle, liver and brain of *Protopterus annectens* fasted in freshwater (control), aestivated in air, or aestivated in mud for 46 days

Tissue	FAA	Content (μmol g^{-1} tissue)		
		46 days in freshwater (control)	46 days aestivation in air	46 days aestivation in mud
Muscle	Aspartate	0.059 ± 0.005	0.32 ± 0.06[a]	0.083 ± 0.015[b]
	Phenylalanine	0.060 ± 0.004	0.032 ± 0.005[a]	0.021 ± 0.008[a]
	Taurine	0.24 ± 0.02	0.21 ± 0.04	0.34 ± 0.02[ab]
	Tyrosine	0.11 ± 0.02	0.20 ± 0.04[a]	0.091 ± 0.009
	TFAA	3.97 ± 1.00	3.70 ± 0.21	3.42 ± 0.19
Liver	Alanine	0.19 ± 0.03	0.10 ± 0.03[a]	0.035 ± 0.013[a]
	Arginine	0.27 ± 0.02	0.33 ± 0.05	0.15 ± 0.02[ab]
	Glutamate	1.58 ± 0.36	0.66 ± 0.11[a]	0.45 ± 0.02[a]
	Glutamine	0.11 ± 0.03	0.014 ± 0.009[a]	0.016 ± 0.006[a]
	Histidine	0.047 ± 0.007	0.12 ± 0.02[a]	0.076 ± 0.003[b]
	Lysine	1.20 ± 0.15	0.76 ± 0.07[a]	0.55 ± 0.05[a]
	Serine	1.05 ± 0.32	0.12 ± 0.02[a]	0.085 ± 0.024[a]
	Taurine	0.33 ± 0.07	0.96 ± 0.17[a]	0.66 ± 0.20
	TFAA	7.21 ± 0.89	4.45 ± 0.37[a]	3.15 ± 0.18[a]
Brain	Alanine	0.097 ± 0.009	0.046 ± 0.011 (3)[a]	0.066 ± 0.012
	Arginine	0.14 ± 0.01	0.088 ± 0.014 (3)[a]	0.093 ± 0.005[a]
	Histidine	0.033 ± 0.006	0.048 ± 0.006 (3)	0.027 ± 0.001[b]
	Serine	0.65 ± 0.09	0.25 ± 0.03 (3)[a]	0.27 ± 0.01[a]
	Taurine	0.21 ± 0.02	0.72 ± 0.10 (3)[a]	0.31 ± 0.02[b]
	Tyrosine	0.058 ± 0.013	0.16 ± 0.03 (3)[a]	0.055 ± 0.004[b]
	Valine	0.070 ± 0.008	0.099 ± 0.005 (3)[a]	0.066 ± 0.008[b]
	TFAA	9.02 ± 0.15	11.1 ± 1.8 (3)	7.55 ± 0.55

Values are means ± SEM (*N*=4 unless state in parentheses).

[a] Significantly different from the corresponding control condition ($p<0.05$).

[b] Significantly different from the corresponding aestivation condition ($p<0.05$).

Fig. 5.1. Rates (μmol day^{-1} g^{-1} fish) of ammonia (☐) and urea (▩) excretion of *Protopterus annectens* during 46 days of fasting in water. Values are means ± S.E.M. (*N*=5). [a]Significantly different from the corresponding day 1-6 ($p<0.05$); [b]Significantly different from the corresponding day 7-12 ($p<0.05$); [c] Significantly different from the corresponding day 13-18 ($p<0.05$).

Fig. 5.1.

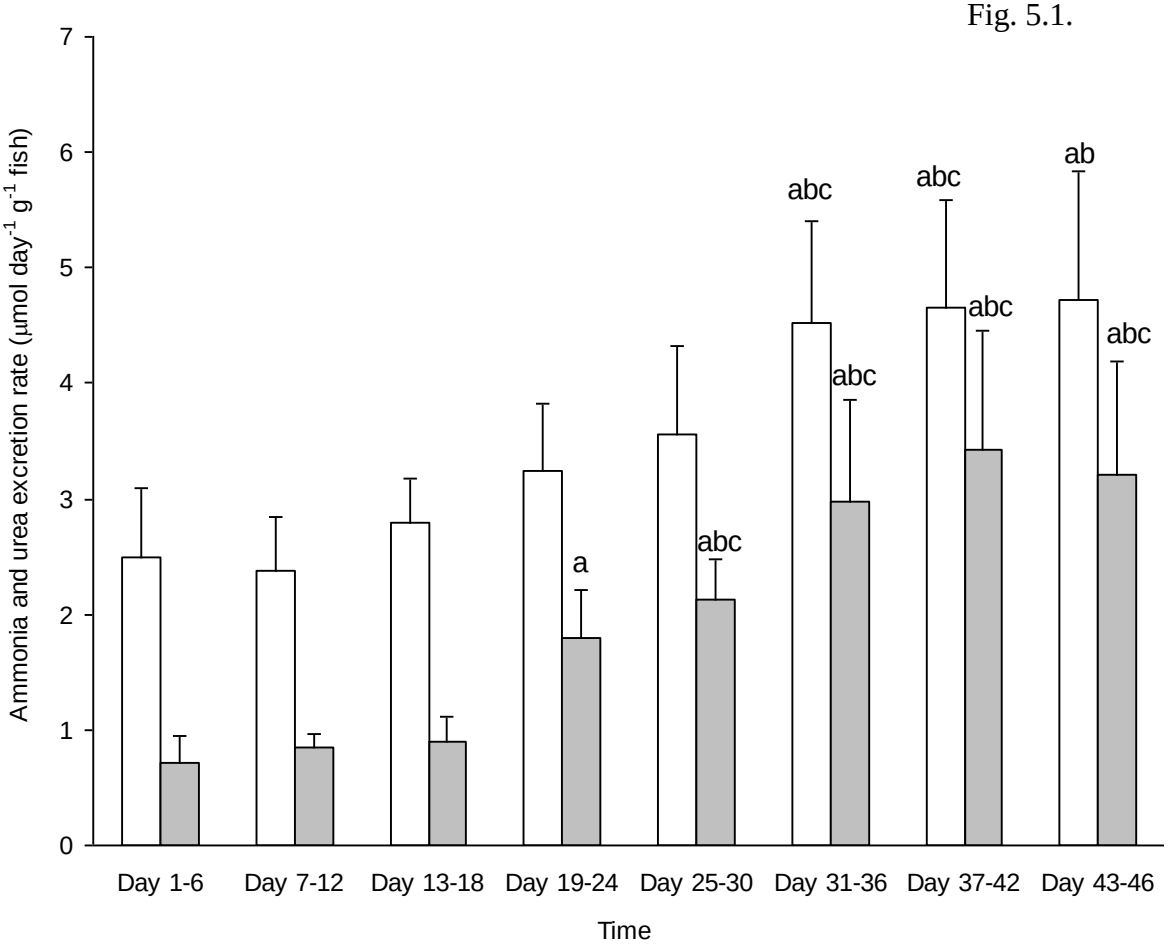

Ammonia and urea excretion rate (µmol day-1 g-1 fish)
0
1
2
3
4
5
6
7
a
abc
abc
abc
abc
abc
ab
Day 1-6
Day 7-12
Day 13-18
Day 19-24
Day 25-30
Day 31-36
Day 37-42
Day 43-46
Time

Fig. 5.2. Contents (μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma) of (a) ammonia and (b) urea in the muscle (□), liver (▒), brain (■) and plasma (▨) of *Protopterus annectens* fasted in freshwater (control) or aestivated in air or mud for 12 days; Values are mean ± S.E.M. (*N*=5). [a]Significantly different from the fasting control in freshwater ($p<0.05$); [b]Significantly different from fish aestivating in air ($p<0.05$).

Fig. 5.2.

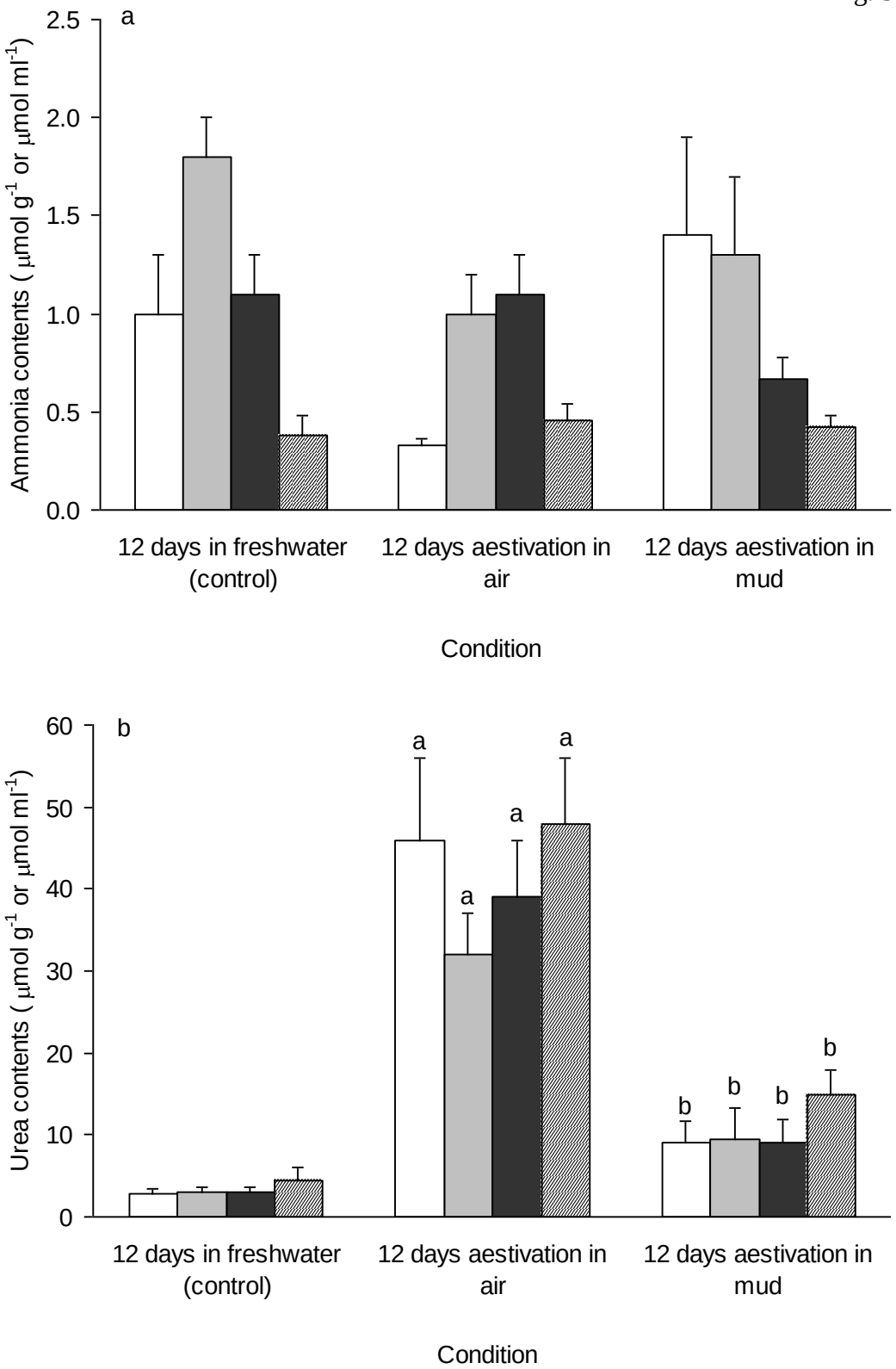
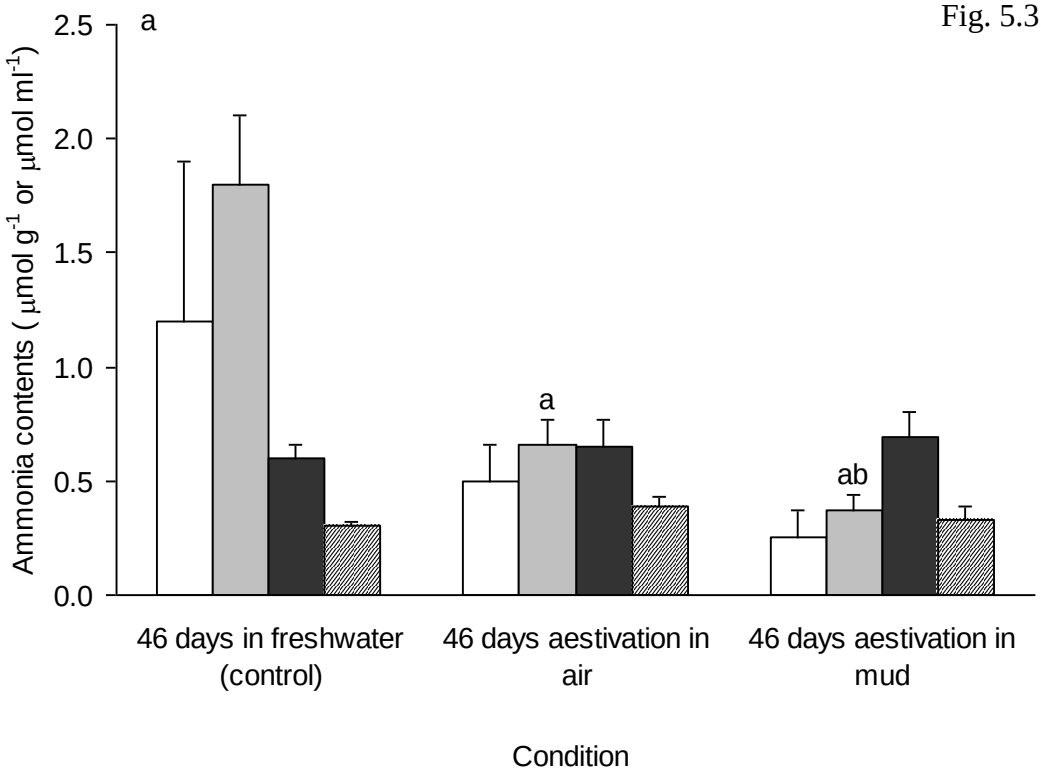

a
Ammonia contents (μmol g-1 or μmol ml-1)
2.5
2.0
1.5
1.0
0.5
0.0
12 days in freshwater (control)
12 days aestivation in air
12 days aestivation in mud
Condition
b
Urea contents (μmol g-1 or μmol ml-1)
60
50
40
30
20
10
0
a
a
a
a
b
b
b
b
12 days in freshwater (control)
12 days aestivation in air
12 days aestivation in mud
Condition

Fig. 5.3. Contents (μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma) of (a) ammonia and (b) urea in the muscle (□), liver (▒), brain (■) and plasma (▨) of *Protopterus annectens* fasted in freshwater (control) or aestivated in air or mud for 46 days; Values are mean ± S.E.M. (*N*=5). [a]Significantly different from the fasting control in freshwater ($p<0.05$); [b]Significantly different from fish aestivating in air ($p<0.05$).


a
2.5
2.0
1.5
1.0
0.5
0.0
Ammonia contents (μmol g-1 or μmol ml-1)
a
ab
46 days in freshwater (control)
46 days aestivation in air
46 days aestivation in mud
Condition

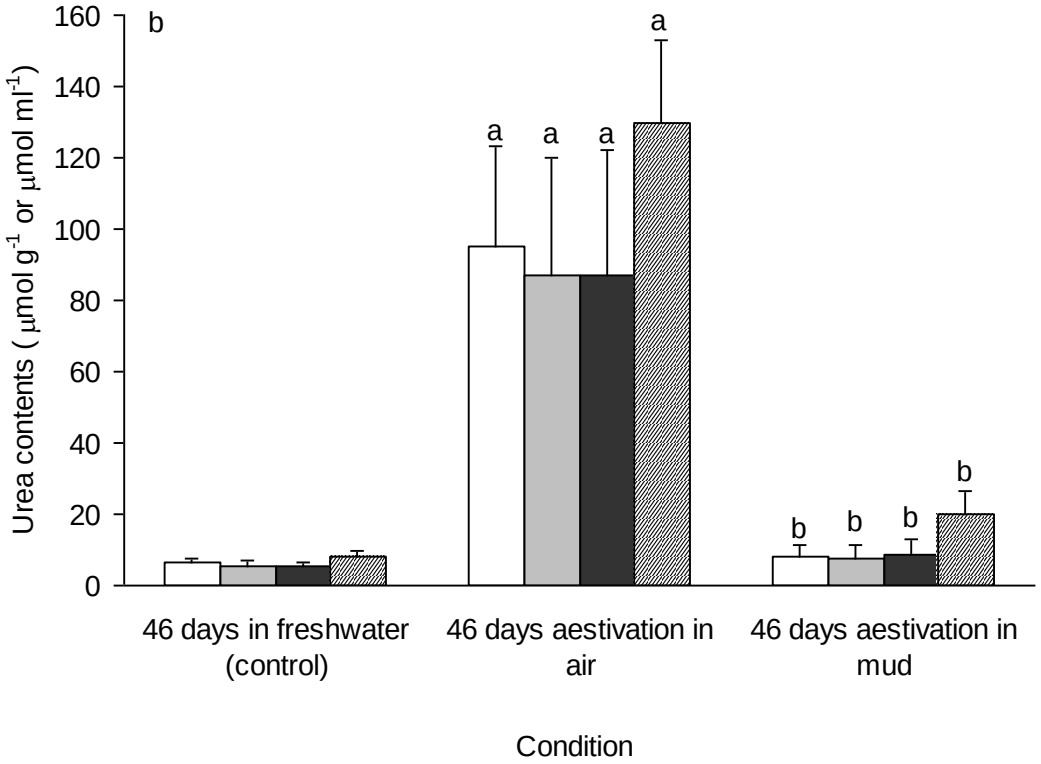

b
160
140
120
100
80
60
40
20
0
Urea contents (μmol g-1 or μmol ml-1)
a
a
a
a
b
b
b
b
46 days in freshwater (control)
46 days aestivation in air
46 days aestivation in mud
Condition

Fig. 5.3.

5.4. Discussion

5.4.1. Effects of fasting (control fish)

The increases in tissue urea contents in fish fasted for 46 days indicate that an increase in ammonia production through increases in protein and amino acid catabolism, probably for energy production, could have occurred. However, despite being ammonotelic (Loong et al. 2005), *P. annectens* detoxified the excess ammonia to urea, while maintaining the tissue ammonia contents unchanged. Since fasting is one of the many factors that could induce aestivation (Fishman et al., 1987) and it also leads to the accumulation of urea in *P. dolloi* (Chew et al. 2004) and *P. annectens* (this study), urea accumulation could be an important signal for the induction of aestivation. Indeed, an injection of urea into the peritoneal cavity of *P. dolloi* led to a suppression of endogenous ammonia production and a decrease in the brain tryptophan content (Ip et al. 2005d), which are important facets of the aestivation process (Chew et al. 2003b, 2004).

5.4.2. Effects of 12 days of aestivation in air

Chew et al. (2004) first reported that *P. dolloi* could aestivate in a completely dried mucus cocoon in air. Subsequently, Wood et al. (2005b) and Perry et al. (2008) worked on fish aestivating in exactly the same conditions in Singapore. However, Wood et al. (2005b; experiment series 1 in that study) described those fish as undergoing aestivation while Perry et al. (2008) describe those as "terrrestrialization". Thus, confusion arises over the terms "aestivation" and "terrestrialization" in the recent lungfish literature. "Terrestrialization" was originally used by Wood et al. (2005b; experiment series 2 in that study) to describe the conditions of *P. dolloi* being exposed to air without the formation of a cocoon, which Chew et al. (2003b) regarded as simply "aerial exposure". In those experiments, water was sprayed directly onto the fish daily to prevent the formation of a cocoon. Subsequently, Wilkie et al. (2007) used "terrestrialization" to describe a state of prolonged exposure of *P. dolloi* to air

during which dried cocoon material was formed along the dorsal, but not the ventral, surface. In that study, water was added to the bottom of the container to prevent the formation of a complete cocoon in order to examine ion fluxes through the ventral surface of the fish (Wilkie et al., 2007). Since aestivation is an adaptation in response to desiccation, and ion and water fluxes could not have occurred after the formation of a completely dried mucus cocoon, "terrestrialization" cannot be regarded as equivalent to "aestivation". It is therefore important to note that, similar to previous studies on other African lungfish species (Chew et al., 2004; Ip et al, 2005f), specimens of *P. annectens* were in fact aestivating in completely dried mucus cocoons in air in this study.

Since *P. annectens* was encased in the cocoon, ammonia excretion would have been completely impeded, which should theoretically result in an accumulation of ammonia, during aestivation. Surprisingly, there was no accumulation of ammonia in tissues of fish aestivated in air for 12 days. Ammonia was effectively detoxified to urea, and urea accumulated in the body instead of ammonia. Since 2483 μmol of urea would have accumulated in the body of a 100 g fish after 12 days of aestivation in air, the average urea synthesis rate during these 12 days can be calculated as 2483/(12 days x 100 g) or 2.07 μmol day^{-1} g^{-1}. This implies an increase of 2.7-fold in comparison with the rate of urea synthesis in the control fish (0.78 μmol day^{-1} g^{-1} fish).

Based on an ammonia excretion rate of 4.0 μmol day^{-1} g^{-1}, a total of 4790 μmol N (calculated from Fig. 5.1) had to be accounted for in a 100 g fish during 12-days of aestivation in air (Table 5.1) assuming that there was no nitrogenous waste excretion. In fact, the urea-N accumulated in various tissues of a 100 g fish amounted to 4966 μmol N, which exceeded slightly the deficit of 4790 μmol N in nitrogenous excretion (Table 5.1), indicating that ammonia was produced at a normal rate but completely detoxified to urea. Since the tissue ammonia contents remained unchanged, the rate of endogenous ammonia production can be calculated as 4966/(100 x 12) or 4.1 μmol day^{-1} g^{-1}, which is indeed comparable to the

value of 4.0 μmol day^{-1} g^{-1} of the day 0 fish. Hence, 12 days of aestivation in air had minimal effects on the estimated rate of ammonia production in *P. annectens*.

Activities of GS and OUC enzymes from the liver of *P. annectens* were unaffected by 12 days of aestivation in air, but CPS III, the rate-limiting enzyme in the OUC, is known to be under allosteric control. Arginine is a powerful activator of N-acetylglutamate synthetase (Shigesada and Tatibana, 1978), and N-acetylglutamate, an activator of CPS III, is the product of the reaction catalysed by N-acetylglutamate synthetase (Campbell and Anderson, 1991). In addition, glutamine is the substrate for CPS III in the reaction that produces carbamoyl phosphate, which is a substrate needed for urea synthesis. Since there were a significant increase and a significant decrease in the contents of arginine and glutamine, respectively, in the liver of *P. annectens* aestivated for 12 days in air, there could indeed be an increase in urea synthesis which prevented ammonia accumulation during this period. The channelling of glutamine to urea synthesis could have resulted in a significant decrease in the glutamate level in the liver.

By contrast, significant increases in glutamate and glutamine contents in the brain of fish aestivated in air for 12 days suggest that the brain, which is incapable of synthesizing urea, was transiently confronted with ammonia toxicity. Besides the detoxification of ammonia to glutamate and glutamine, there could also be a decrease in amino acid catabolism which led to a reduction in ammonia production and accumulations of some FAAs in the brain. Similar phenomena have been reported for *P. dolloi* during 6 days of aestivation in air (Chew et al., 2004), but the contents of glutamine and glutamate build up to higher levels in *P. annectens* than *P. dolloi*, indicating the prowess of the latter in decreasing ammonia production.

5.4.3. Effects of 46 days of aestivation in air

Protopterus annectens had a greater dependency on decreased ammonia production than increased urea synthesis to ameliorate ammonia toxicity during 46 days of aestivation in air because there was a significant decrease in the ammonia content in the liver, and the deficit in nitrogenous excretion during this period could not be completely accounted for by the excess urea accumulated. Since excess urea amounted to 5188 μmol in a 100 g fish, the average urea synthesis rate during these 46 days is 5188/(46 days x 100 g) or 1.1 μmol day^{-1} g^{-1} (Table 5.1), meaning that the estimated rate of urea synthesis increased 1.41-fold during this period (in comparison with 0.78 μmol day^{-1} g^{-1}). The increased rate of urea synthesis did not exceed the OUC capacity, and thus activities of hepatic OUC enzymes remained unchanged.

Assuming the absence of nitrogenous waste excretion, the deficit in nitrogenous excretion during the 46-day period can be calculated as 34028 μmol N for a 100 g fish (from Fig. 5.1), but the urea-N accumulated in various tissues amounted to 10376 μmol only (Table 5.1). Thus, unlike fish aestivated in air for 12 days, the production of ammonia through amino acid catabolism in fish aestivated in air for 46 days was reduced to 31% of that in the fasted control. Additionally, the rate of ammonia production can be estimated as 10376 μmol/(100 g x 46 days) = 2.26 μmol day^{-1} g^{-1}, which is only 56% of the day 0 value (4.0 μmol day^{-1} g^{-1}). Furthermore, there could be a decrease in protein degradation in the liver, the magnitude of which was greater than that of the decrease in amino acid catabolism, leading to a significant decrease in the liver TFAA content in fish aestivated in air for 46 days. Consequently, the brain of these fish was no longer exposed to ammonia toxicity as reflected by unchanged contents of glutamine, glutamate and TFAA therein.

5.4.4. Effects of 12 days of aestivation in mud

By contrast, aestivation in mud for 12 days did not result in significant increases in tissue urea contents in *P. annectens*. Since only 366 μmol of excess urea accumulated in a

100 g fish, the estimated urea synthesis rate during this period was 366/(12 days x 100 g) or 0.31 μmol day^{-1} g^{-1} (Table 5.1). Thus, the average rate of urea synthesis decreased to 0.31/0.78 or 40% of the day 0 value. This is distinctly different from fish aestivated in air for 12 days, of which the urea synthesis rate increased 2.7-fold.

The urea-N accumulated in various tissues of a 100 g fish aestivated in mud for 12 days amounted to 366 x 2 = 732 μmol N, which is much lower than the deficit of 4790 μmol N in nitrogenous excretion (Table 5.1). Therefore, unlike aestivation in air, aestivation in mud resulted in a decrease in endogenous ammonia production in *P. annectens*. The rate of ammonia production decreased to 732/4790 or 15% of the fasting control during this period. The average rate of ammonia production can be calculated as 732 μmol/(100 g x 12 days) or 0.61 μmol day^{-1} g^{-1}, which indicates that it had decreased to 15% of the day 0 value (4.0 μmol day^{-1} g^{-1}). The decrease in ammonia production was probably achieved through a reduction in amino acid catabolism in general, which should theoretically lead to increases in contents of FAAs and TFAA in the muscle and/or liver if the rate of protein degradation remained unchanged. Since such increases were either minor or did not occur, it can be deduced that the reduction in ammonia production was accompanied with a decrease in protein degradation. Unlike fish aestivating in air for 12 days, the decrease in ammonia production in extra-cranial tissues in fish aestivating in mud for 12 days effectively prevented the brain from ammonia toxicity and thus avoided the accumulation of glutamate/glutamine.

Taken altogether, these results indicate that *P. annectens* was able to vary its physiological responses during aestivation in air or in mud. In air, where oxygen is available, it increased the rate of urea synthesis to detoxify ammonia. However, in mud, where oxygen is limiting, it drastically decreased ammonia production to ameliorate ammonia toxicity, which can be viewed as an adaptation to reduce energy expenditure through urea synthesis.

5.4.5. Effects of 46 days of aestivation in mud

Surprisingly, the excess urea accumulated in a 100 g fish during 46 days of aestivation in mud amounted to only 111 μmol, despite the large deficit in nitrogenous excretion (34028 μmol N for a 100 g fish). The average urea synthesis rate during this period can be estimated as 111 μmol/(46 days x 100 g) or 0.02 μmol day^{-1} g^{-1} (Table 5.1), which decreased to 0.02/0.78 or a meagre 2.6% of the value for fish kept in freshwater. Thus, it is confirmed that increased urea synthesis was not a major adaptation to defend against ammonia toxicity in *P. annectens* during 46 days of aestivation in mud. The urea-N accumulated in various tissues amounted to 111 μmol x 2 = 222 μmol N, which is much smaller than the deficit of 34028 μmol N in nitrogenous excretion (Table 5.1). This is the first report of such a phenomenon in African lungfishes aestivating in mud. Since previous works on African lungfishes involved a longer time period of aestivation in artificial mud cocoons (i.e. 78 days to 6 months or more; Smith, 1930; Janssens, 1964; Janssens and Cohen, 1968a), the magnitudes of urea accumulation presented in those reports were greater than those reported herein. However, the possibility of a reduction in the rate of urea synthesis during the aestivation period were neglected in those studies because data had not been analyzed quantitatively, taking the period of aestivation into consideration.

From these results, it can be concluded that fish decreased the production of endogenous ammonia drastically through a reduction in amino acid catabolism during 46 days of aestivation in mud. Since there was a significant decrease in the TFAA content in the liver, a decrease in protein degradation could have occurred, and the magnitude of decrease in protein degradation must be greater than the magnitude of decrease in amino acid catabolism. From the above calculations (222 μmol N/34028 μmol N), it is confirmed that the estimated rate of ammonia production decreased to 0.65% of the fasted control, and this could be the greatest reduction in ammonia production known in animals. The rate of ammonia production calculated as 222 μmol N/ (100 g x 46 days) or 0.048 μmol day^{-1} g^{-1} indicate that it had decreased to 1.2% of the value (4.0 μmol day^{-1} g^{-1}) obtained for the day 0 fish in water.

With such a profound suppression of ammonia production, it is no wonder that African lungfishes can aestivate in mud cocoon for 3 to 5 years (Smith, 1930, 1935).

5.4.6. Why would P. annectens depend more on decreased ammonia production than increased urea synthesis to ameliorate ammonia toxicity during 46 days of aestivation in mud?

There could be two possible reasons why *P. annectens* sustained a high rate of urea synthesis during the initial 12 days of aestivation in air, although it was energy-intensive to do so. Firstly, the fish aestivated in a thin layer of dried mucus in open air with high O_2 tension; thus, it remained aerobic and there were no changes in the blood pO_2 and muscle ATP content after 46 days. Secondly, aestivation in air entailed desiccation as reflected by a decrease in the wet mass of the fish, and therefore increased tissue urea contents might serve the secondary function of facilitating water retention through vapour pressure depression. However, the second possibility was subsequently invalidated by results presented in Chapter 6 of this thesis. Hence, it would be logical to deduce that urea accumulation was essential to prevent water loss as the external medium dried up during the induction phase (i.e. before the formation of a complete cocoon and the ventral body surface was in direct contact with the external medium) and the initial maintenance of aestivation in air.

By contrast, fish aestivating in mud for 12 or 46 days did not adopt urea synthesis as a strategy to ameliorate ammonia toxicity. The rate of dehydration through desiccation was greatly reduced in mud as reflected by the lack of changes in body masses of fish before and after aestivation therein. Thus, there was no necessity for *P. annectens* to accumulate urea to facilitate water retention within this short period, and it could depend mainly on a reduction in ammonia production to ameliorate ammonia toxicity during 46 days of aestivation in mud. However, it is probable that urea would accumulate to high levels in fish aestivating in mud

for an extended period (Smith, 1930; Janssens, 1964; Janssens and Cohen, 1968a), because ammonia is being produced continuously, albeit at low rates, which would result in a slow but sustained increase in tissue urea contents. More importantly, fish aestivating in mud were exposed to environmental hypoxia as reflected by decreases in blood pO_2 and muscle ATP content, which are in agreement with a previous report on *P. aethiopicus* aestivating in subterranean cocoons (DeLaney et al., 1974). Indeed, by inducing *P. annectens* to aestivate in sealed plastic containers continuously flushed with either air (normoxia) or 2% O_2 in N_2 (hypoxia), we have confirmed that the rate of urea accumulation in fish aestivating in hypoxia for 12 days was significantly lower than those aestivating in normoxia for a similar period (see Chapter 3). Hence, similar to the swamp eel, *M. albus*, (Chew et al., 2005), hypoxia could be instrumental to initiating a profound decrease in the rate of ammonia production in *P. annectens* aestivating in mud, and it is therefore important to study the intricate relationships between ammonia tolerance and hypoxia tolerance in these tropical air-breathing fishes in the near future.

5.4.7. Aestivation in air versus aestivation in mud

Chew et al. (2004) reported that *P. dolloi* accumulated urea in its tissues after 6 or 40 days of aestivation in air, but the magnitudes of urea accumulation were lower than those observed for *P. annectens* aestivating in air in this study. During 6 days of aestivation in air, *P. dolloi* could reduce its endogenous ammonia production, but *P. annectens* was apparently incapable of doing so. Therefore, it can be deduced that *P. dolloi* is better adapted to aestivate in air than *P. annectens*, because the former can suppress ammonia production more effectively than the latter. Hence, in nature, *P. annectens* and *P. dolloi* may prefer to aestivate in subterranean cocoons and dried mucus cocoon in air, respectively, despite the fact that

both can be induced to aestivate in air or in mud in the laboratory. If indeed that is the case, it would explain why the subterranean cocoon of *P. annectens* becomes a common example of aestivation in lungfishes in the literature but no subterranean cocoon of *P. dolloi* has been found in nature so far (Greenwood, 1987). It can be envisaged that aestivating in air has the disadvantages of exposure to desiccation and predation, but it offers *P. dolloi* the advantages of a normoxic environment, efficient arousal from aestivation once water becomes available, and consequently short aestivation periods. On the other hand, aestivating in mud would offer the advantages of avoidance of desiccation and predation, but *P. annectens* would have to overcome the disadvantages of hypoxic exposure, difficulties in responding to water when it becomes available which may result in long periods of aestivation, and difficulties in emergence from dried mud. In our laboratories, fish aestivating in a mucus cocoon in air can be aroused simply by the addition of water, but fish aestivating in mud cannot be aroused by flooding the mud surface with water unless the dried mud was mechanically broken down into small pieces to expose the fish to water (Y. K. Ip, S. F. Chew and A. M. Loong, unpublished observations).

5.5. Summary

The objective of this study was to elucidate how the African lungfish, *Protopterus annectens*, ameliorated ammonia toxicity during 12 or 46 days of aestivation in air or in mud. Twelve days of aestivation in air led to significant increases in contents of urea, but not ammonia, in tissues of *P. annectens*. The estimated rate of urea synthesis increased 2.7-fold despite the lack of changes in the activities of hepatic ornithine-urea cycle enzymes, but there was only a minor change in the estimated rate of ammonia production. After 46 days of aestivation in air, the ammonia content in the liver decreased significantly and contents of urea in all tissues studied increased significantly, indicating that the fish shifted to a combination of increased urea synthesis (1.4-fold of the day 0 value) and decreased ammonia production (56% of the day 0 value) to defend against ammonia toxicity. By contrast, 12 days of aestivation in mud produced only minor increases in tissue urea contents, with ammonia contents remained unchanged. This was apparently achieved through decreases in urea synthesis and ammonia production (40% and 15%, respectively, of the corresponding day 0 value). Surprisingly, 46 days of aestivation in mud resulted in no changes in tissue urea contents, indicating that profound suppressions of urea synthesis and ammonia production (2.6% and 1.2%, respectively, of the corresponding day 0 value) had occurred. This is the first report on such a phenomenon, and the reduction in ammonia production was so profound that it could be the greatest reduction known among animals. Since fish aestivated in mud had relatively low blood pO_2 and muscle ATP content, they could have been exposed to hypoxia, which induced reductions in metabolic rate and ammonia production. Consequently, fish aestivating in mud had a lower dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, than fish aestivating in air.

6. Chapter 3:

Effects of normoxia versus hypoxia (2% O_2 in N_2) on the energy status and nitrogen metabolism of *Protopterus annectens* during aestivation in a mucus cocoon

6.1. Introduction

Lungfishes, as members of Class Sarcopterygii, are well-known for their plausible involvement in water-land transition during evolution. There are six species of extant lungfishes, four of which can be found in Africa. African lungfishes, belonging to Family Protopteridae, possess two lungs and are obligatory air-breathers (Graham, 1997). They can often be found in hypoxic waters. Unlike their South American and Australian counterparts, African lungfishes undergo aestivation in the absence of water during drought, and remain incarcerated in this state of inactivity until the return of water to the habitat (Fishman et al., 1987; Ip et al. 2005f). They can aestivate inside a cocoon made of dried mucus in air (*Protopterus dolloi*, Chew et al., 2004; *Protopterus aethiopicus*, Ip et al., 2005f; *Protopterus annectens*, Loong et al., 2008b) or burrow into the mud and aestivate in a subterranean cocoon (*Protopterus annectens* and *P. aethiopicus*; Janssens, 1964; Janssens and Cohen, 1968a, b; Loong et al. 2008b).

African lungfishes are ureogenic; they possess a full complement of ornithine-urea cycle (OUC) enzymes (Janssens and Cohen, 1966, 1968a; Mommsen and Walsh, 1989), including carbamoyl phosphate synthetase III (CPS III), in their livers (Chew et al., 2003b; Loong et al., 2005). However, they are ammonotelic in water (Lim et al., 2004; Loong et al., 2005; Ip et al., 2005f). During aestivation, ammonia excretion would be impeded, leading to its accumulation in the body. Since ammonia is toxic (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip et al., 2001; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002), African lungfishes have to avoid ammonia toxicity during aestivation, and they achieve this through an increase in urea synthesis (Smith, 1930, 1935; Janssens, 1964; Janssens and Cohen, 1968a, b) and a suppression of ammonia production (see Ip et al., 2004a; Chew et al., 2006 for reviews). Recently, Chew et al. (2004) demonstrated that the rate of urea synthesis increased 2.4- to 3.8-fold and the rate of ammonia production decreased by 72% in *P. dolloi* during 40 days of aestivation in air (normoxia) when compared with the immersed control.

Urea synthesis is energy intensive; 5 mol of ATP are required for the formation of one mole of urea. Therefore, increased urea synthesis may not be an effective adaptation in fish aestivating in hypoxic mud, as environmental hypoxia causes a low efficiency of ATP production due to the exploitation of anaerobic pathways (Hochachka, 1980). Indeed, results presented in the Chapter 2 of this thesis (Loong et al., 2008b) reveal that 46 days of aestivation in mud resulted in no changes in tissue urea concentrations in *P. annectens*, which indicates that profound suppressions of urea synthesis and ammonia production had occurred. Since fish aestivating in mud had low blood pO_2 and muscle ATP concentrations, the author (Loong et al., 2008b) speculated that they could have been exposed to hypoxia, resulting in greater reductions in metabolic rate and ammonia production. Consequently, there was a lower dependency on increased urea synthesis to detoxify ammonia in the fish aestivating in mud as compared with those aestivating in air. Therefore, this study was undertaken to evaluate and compare effects of normoxia and hypoxia on tissue energetics and nitrogen metabolism in *P. annectens* during induction (days 3 and 6) or maintenance (day 12) of aestivation under laboratory conditions. On day 3, the fish was exposed to air and on day 6 the fish would have entered into aestivation with the formation of a completely dried mucus cocoon. In our laboratory, fish can aestivate in a mucus cocoon in air for at least 1 year. Thus, contrary to the proposition of Perry et al. (2008), these experimental fish cannot be regarded as undergoing "terrestrialization", because no water was added to prevent the formation of a completely dried cocoon as in the case of series 2 experiment performed by Wood et al. (2005b). Since the author could induce *P. annectens* to aestivate in air-tight plastic boxes, she was able to determine for the first time ATP and creatine phosphate concentrations in various regions of the live fish during 12 days of induction and maintenance of aestivation using *in vivo* ^{31}P NMR spectroscopy.

Additionally, the author determined tissue ammonia and urea concentrations of fish aestivating in normoxia or hypoxia in order to test the hypothesis that the magnitude of

increase in urea synthesis and accumulation would be lower in fish aestivating in hypoxia than in normoxia. Traditionally, it has been assumed that metabolic rate reduction naturally occurs in African lungfishes in association with aestivation but without differentiating whether aestivation takes place in hypoxia or normoxia (Smith, 1935; Janssens and Cohen, 1968a, b). However, it has been demonstrated that *P. dolloi* aestivating in a completely dried mucus cocoon in air (normoxia) had a respiratory rate comparable to that of control fish immersed in water (Perry et al., 2008), and the respiratory rate of fish immersed in water was greatly reduced by aerial hypoxia (Perry et al., 2005a). The author therefore reasoned that there could be a greater reduction in metabolic rate in fish aestivating in hypoxia than in normoxia, resulting in a greater suppression in nitrogen metabolism in the former than in the latter. Hence, the concentrations of free amino acids (FAAs) in various tissues were determined in order to deduce indirectly whether there was a larger decrement of amino acid catabolism in fish exposed to hypoxia than to normoxia.

Finally, to confirm that aestivation in hypoxia indeed affected amino acid metabolism in *P. annectens*, the author examined, for the first time, the kinetic properties of glutamate dehydrogenase (GDH), in both amination and deamination directions, from livers of the normoxic and hypoxic fish. GDH catalyzes the amination of α-ketoglutarate in the presence of NADH or the deamination of glutamate in the presence of NAD. Glutamate formed by the amination reaction can act as a substrate for transamination of amino acids or the formation of glutamine, which is the substrate of urea synthesis in the hepatic ornithine-urea cycle (Chew et al., 2003b; Loong et al., 2005). Conversely, α-ketoglutarate produced through glutamate deamination can be shuttled into the tricarboxylic acid cycle for ATP production. Hence, GDH is in a crucial position to regulate the degradation of amino acids and plays an important role in integrating nitrogen and carbohydrate metabolism. Additionally, GDH is known to be activated by ADP (Campbell, 1973), the concentration of which may change during hypoxic exposure, and GDH can also be modified by ADP-ribosylation (Herrero-

Yraola et al., 2001). Thus, the author aimed to test two hypotheses: (1) there could be changes in specific activity and kinetic properties of GDH, in amination and/or deamination directions, from the liver of *P. annectens* during the induction and maintenance phases of aestivation, and (2) these changes might be different between normoxic and hypoxic fishes, especially with regard to ADP activation *in vitro*. Since Richardson's ground squirrel (*Spermophilus richardsonii*) possesses two distinct forms of GDH and since the GDH properties change during hibernation (Thatcher and Storey, 2001), the author aimed to deduce indirectly from the kinetic properties of hepatic GDH from *P. annectens* whether different forms of GDH existed in this African lungfish.

6.2. Materials and methods

6.2.1. Fish

Protopterus annectens (80-120 g body mass) were imported from Central Africa through a local fish farm in Singapore. Specimens were maintained in plastic aquaria filled with dechlorinated water, containing 2.3 mmol l^{-1} Na^+, 0.54 mmol l^{-1} K^+, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^- and 0.6 mmol l^{-1} HCO_3^-, at pH 7.0 and at 25°C in the laboratory, and water was changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 month. During the adaptation period, fish were fed frozen fish meat. In June 2005 and June 2006, fish were transported to Düsseldorf and then to Bremerhaven under animal experimentation Permit (50.05-230-44/05, Landesamt für Natur, Umwelt und Vebraucherschutz, NRW) for ^{31}P NMR studies.

We succeeded in inducing *P. annectens* to aestivate in the presence of small volumes of water inside air-tight plastic containers continuously flushed with air or a calibrated gas mixture (2% O_2 in N_2). With such a set up, we overcame problems associated with controlling the severity and consistency of hypoxic exposure as in the case of experimenting with fish aestivating in mud (as in its natural habitat; Chapter 2; Loong et al., 2008b). In addition, we eliminated problems associated with the interference of ^{31}P NMR application by mud. Under standard laboratory conditions, the experimental fish would secrete mucus during the first few days, and the mucus would slowly dry up between day 6 and day 7 to form a mucus cocoon. Therefore, three major time points were defined in this study, i.e. day 3 (preparation for aestivation), day 6 (entering into aestivation) and day 12 (after 5-6 days of aestivation), with additional time points for the *in vivo* ^{31}P NMR spectroscopy.

6.2.2. Determination of ATP and creatine phosphate concentrations at three different regions of live fish using in vivo *^{31}P NMR spectroscopy*

Normoxic fish were exposed individually to terrestrial conditions and allowed to enter into aestivation at 23^{o}C in air-tight plastic containers (17.5 cm x 11.5 cm x 5 cm, length x width x height) containing 20 ml of water. The head space of boxes was flushed continuously (50 ml min^{-1}) with air (20.9% O_2 in N_2) for 12 days. Hypoxic fish underwent aestivation in similar plastic containers but they were flushed with 2% O_2 in N_2 instead. The gas was mixed using a gas-mixing pump (Wösthoff, Bochum, Germany). Control measurements were taken before the fish were exposed to terrestrial conditions (day 0), and measurements continued on days 1, 3, 6, 9 and 12 for each individual fish.

In vivo ^{31}P NMR spectroscopy experiments were conducted using a 47/40 Bruker Biospec DBX system with a 40 cm horizontal wide bore and actively shielded gradient coils (50 mT m^{-1}) (Melzner et al., 2006). A 5 cm ^{1}H/^{31}P/^{13}C surface coil was used for excitation and signal reception. The coil was placed directly under the animal chamber to gain maximum signal from three different regions (anterior, middle and posterior) of the fish. The anterior region of the fish refers to the head; the middle region refers to the location of the liver; and, the posterior region refers to the position before the vent where the kidney is located. It was hoped that results obtained would provide some information on possible changes in the energy status in brain, liver and kidney in addition to possible changes in muscle. Temperature in the animal chamber inside the magnet was kept at 23°C and monitored by a fiber-optic thermometer (Luxtron 504, Polytec, Waldheim, Germany) and recorded via a MacLab system (AD-Instruments, Australia). *In vivo* ^{31}P NMR spectra (sweep width, 5000 Hz; flip angle, 45°, repetition time (TR), 1 s; scans, 256; duration, 4 min 31 s) were acquired and an average of 4 spectra was taken from each region. Concentrations of ATP and creatine phosphate were determined from the NMR spectra according to the method of Kemp et al. (2007) and expressed as µmol g^{-1} wet mass.

6.2.3. Exposure of fish to experimental conditions for tissue sampling

Normoxic fish were individually exposed to air and allowed to enter into aestivation at 25^{o}C in air-tight plastic containers (7.6 cm x 15.7 cm, height x diameter) containing 20 ml of water. The head space was continuously flushed (50 ml min^{-1}) with air (20.9% O_2 in N_2) for 12 days. Hypoxic fish were exposed to aerial hypoxia in similar plastic containers but continuously flushed with 2% O_2 in N_2 instead. Fish were killed on days 3, 6 or 12 with a strong blow to the head. Plasma, lateral muscle, and liver were sampled and kept at -80°C until analysis.

6.2.4. Determination of water content in the muscle and liver

Water contents in muscle and liver samples (*N*=3 each) obtained from control fish and fish aestivated in air or hypoxia for 12 days were estimated as the difference between wet mass and dry mass, and expressed as percent of wet mass tissue. The wet masses of the tissues were recorded to the nearest 0.001 g. The tissues were then dried in an oven at 95ºC until constant mass and the dry mass was recorded.

6.2.5. Determination of ammonia, urea and FAAs

The frozen samples were weighed, ground in liquid nitrogen and homogenized three times in five volumes (w/v) of 6% TCA at 24, 000 revs min^{-1} for 20 sec each using an Ultra-Turrax homogenizer (Staufen, Germany), with intervals of 10 sec between each homogenization. The homogenate was centrifuged at 10, 000 x*g* at 4°C for 20 min, and the supernatant obtained was kept at –80°C until further analysis.

For ammonia urea and FAA analysis, the methods were similar to those presented in Chapter 1, section 4.2.3. The amount of urea accumulated in a hypothetical 100 g fish, which contained 55 g muscle, 2 g liver, 0.3 g brain and 1 ml plasma, was calculated according to method of Loong et al. (2005).

6.2.6. Determination of hepatic GDH enzyme activity

The liver was homogenized in five volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} imidazole (pH 7.0), 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 25 mmol l^{-1} NaF and 0.1 mmol l^{-1} PMSF according to the method of Ip et al. (1992). The homogenate was sonicated for 10 sec and the sonicated sample was centrifuged at 10, 000 x*g* at 4°C for 20 min. The supernatant obtained was passed through a Bio-Rad P-6DG column (Bio-Rad Laboratories; Hercules, CA, USA) equilibrated with the elution buffer containing 50 mmol l^{-1} imidazole (pH 7.0) and 1 mmol l^{-1} EDTA. The filtrate obtained was used directly for enzyme assay.

GDH (E.C. 1.4.1.3) activities were assayed according to methods of Ip et al. (1992, 1994) and Peng et al. (1994) using a Shimadzu UV 160 UV VIS recording spectrometer at 25°C. GDH activity in the amination direction was determined by the oxidation of NADH at 340 nm (millimolar extinction coefficient ε_{340} = 6.22) in a reaction mixture (1.2 ml) containing 50 mmol l^{-1} imidazole buffer (pH 7.4), 250 mmol l^{-1} ammonium acetate, 0.15 mmol l^{-1} NADH, 1.0 mmol l^{-1} ADP and 0.05 ml sample. The reaction was initiated by the addition of 0.05 ml of α-ketoglutarate (α-KG) at a final concentration (mmol l^{-1}) of 0.1, 0.25, 0.5, or 10. The activity obtained at 10 mmol l^{-1} α-KG was regarded as $V_{control}$ (approaching V_{max}). The amination activity was expressed as μmol NADH oxidized min^{-1} g^{-1} wet mass. GDH activity in the deamination direction was determined by measuring the formation of formazan from iodonitrotetrazolium chloride at 492 nm (millimolar extinction coefficient ε_{492} = 19.98) in a reaction mixture (1.35 ml) containing 200 mmol l^{-1} glycine-NaOH buffer (pH 9.0), 0.1 mmol l^{-1} NAD, 0.09 mmol l^{-1} iodonitrotetrazolium chloride, 0.1 iu/ml diaphorase, 1.0 mmol l^{-1} ADP and 0.15 ml sample. This reaction was initiated by the addition of 0.1 ml of glutamate at a final concentration (mmol l^{-1}) of 0.5, 5 or 100. The activity obtained at 100 mmol l^{-1} glutamate was regarded as $V_{control}$. The deamination activity

was expressed as μmol formazan formed min^{-1} g^{-1} wet mass. In addition, amination activities at 10 mmol l^{-1} α-KG and deamination activity at 100 mmol l^{-1} glutamate were also determined in the absence of ADP ($V_{minus\ ADP}$). All chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Due to the small size of the liver and the various assays need to be performed, the volume of extract obtained for GDH assay was inadequate for the estimation of Km or Ka values, which required the determination of GDH activities at multiple substrate or activator (ADP) concentrations. Therefore, we adopted the method of expressing the results as activity ratios, which had been utilized previously by Ip et al. (1994) and Peng et al. (1994) to examine the effects of anoxia and salinity stress, respectively, on the kinetic properties of GDH from the intertidal spicunculid, *Phascolosoma arcuatum*. This method was originally designed by Plaxton and Storey (1985) to examine the effect of hypoxia on the kinetic properties of pyruvate kinase from the whelk, *Busycotypus canaliculatum*. In that study, a significantly greater enzyme activity ratio, measured at high versus low phosphoenolpyruvate concentration obtained from the normoxic animal as compared with the hypoxic animal, was taken as an indication of an increase in $S_{0.5}$ of phosphoenolpyruvate for the anoxic form of pyruvate kinase. (Plaxton and Storey, 1985).

6.2.7. Determination of ammonia and urea excretion rates in control fish immersed in water

Fish were immersed individually in 20 volumes (w/v) of dechlorinated tap water in plastic aquaria at 25^{o}C without aeration. Water was changed daily and no food was provided. Preliminary experiments on water sampled at 6 and 24 hour showed that ammonia and urea excretion rates were linear up to at least 24 hour. Water (3.6 ml) was sampled for ammonia and urea analysis every 24 hour for 12 days. Ammonia and urea in water samples were determined according to the methods of Jow et al. (1999).

6.2.8. Statistical analyses

Results were presented as means ± S.E.M. Time-course data in Fig. 6.1, and 6.2 were analyzed using 2-way repeated-measures ANOVA followed by Tukey-HSD method to evaluate differences between means. For other data, Student's t-test and one-way analysis of variance (ANOVA) followed by multiple comparison of means by the Bonferroni test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $p<0.05$.

6.3. Results

6.3.1. ATP and creatine phosphate in three different regions of the fish based on ^{31}P NMR spectroscopy

Twelve days of induction and maintenance of aestivation in *P. annectens* in normoxia or hypoxia did not result in significant changes in ATP (Fig. 6.1) or creatine phosphate (Fig. 6.2) concentrations in all three regions of the body. In comparison with normoxia, hypoxia led to significantly lower ATP concentration on day 12 (Fig. 6.1) and also significantly lower creatine phosphate concentration on days 1, 6, 9 and 12 (Fig. 6.2) in the anterior region of fish undergoing induction and maintenance of aestivation. Additionally, hypoxia resulted in a significantly lower creatine phosphate concentration in the middle region of fish undergoing aestivation on day 9.

Since these results were obtained from whole fish, they do not provide information on any specific tissue or organ. However, the detection of significant amount of creatine phosphate in the middle region of the fish, where the liver is located, was unexpected because creatine phosphate is a phosphagen found mainly in the muscle (Prosser, 1973). Hence, either the creatine phosphate concentration obtained for the middle region based on ^{31}P NMR spectroscopy was contributed mainly by the muscle, or the liver actually contained an unusually high concentration of creatine phosphate, the confirmation of which awaits future study.

6.3.2. Water contents in the muscle and liver

The water content (N=3) in the muscle of *P. annectens* after 12 days of induction and maintenance of aestivation in normoxia and hypoxia were 80.1 ± 1.8% and 77.6 ± 2.1%, respectively, which were not significantly different from the value (78.6 ± 1.4%) obtained for the control in freshwater. Similarly, the water contents (N=3) in the livers of control fish

(79.4 ± 0.9%) and fish after 12 days of induction and maintenance of aestivation in normoxia (78.3 ± 0.8%) or hypoxia (77.9 ± 1.1%) were comparable.

6.3.3. Ammonia and urea concentrations

The ammonia concentrations in muscle, liver and plasma of fish kept in freshwater on day 0 were 0.48 ± 0.28 μmol g^{-1}, 1.07 ± 0.35 μmol g^{-1}, and 0.37 ± 0.11 μmol ml^{-1}, respectively, which were not significantly different (statistics not shown) from those values of the experimental fish exposed to normoxia or hypoxia (Table 6.1). There were no significant differences in the ammonia concentrations between the muscle, liver, and plasma of the normoxic fish and the hypoxic fish throughout the 12-day period (Table 6.1).

The urea concentrations in muscle, liver and plasma of fish kept in freshwater on day 0 were 3.18 ± 0.86 μmol g^{-1}, 3.64 ± 1.05 μmol g^{-1}, and 4.08 ± 1.17 μmol ml^{-1}, respectively, which were significantly lower (P<0.05) than those of the experimental fish exposed to normoxia or hypoxia. On days 3 and 6, the urea concentration in the muscle of *P. annectens* exposed to hypoxia remained comparable to that of fish exposed to normoxia (Fig. 6.3a). On day 12, the urea concentration in the muscle of fish aestivating in hypoxia was significantly lower (~50%) than that of the fish aestivating in normoxia (Fig. 6.3a). By contrast, the urea concentration in the liver of fish entering into aestivation in hypoxia on days 3 and 6 was significantly lower (by 44% and 41%, respectively) than that of the fish entering into aestivation in normoxia. However, there was no significant difference in the hepatic urea concentration between the fish aestivating in hypoxia and normoxia on day 12 (Fig. 6.3b). As for the plasma, the urea concentration in fish entering into aestivation in hypoxia was significantly lower than that of fish entering into aestivation in normoxia on day 6 (Fig. 6.3c).

6.3.4. FAA concentrations

Muscle arginine, leucine, phenylalanine and tyrosine concentrations in fish exposed to hypoxia for 3 days, and the muscle tyrosine concentrations in fish exposed to hypoxia for 6 days were significantly higher than the corresponding value of the normoxic fish (Table 6.2). However, concentrations of TFAA and TEFAA in the muscle of the hypoxic fish were comparable with those of the normoxic fish throughout the 12-day period (Table 6.2).

By contrast, concentrations of tyrosine, TEFAA and TFAA in the liver of fish exposed to hypoxia for 3 days were significantly higher than those of fish exposed to normoxia for a similar period (Table 6.2). Similarly, exposure to hypoxia for 6 days resulted in significantly higher concentrations of alanine, glutamate, proline and TFAA in the liver as compared with the corresponding normoxic values (Table 6.2). There were significantly higher glutamate, tryptophan and TEFAA concentrations in the liver of fish aestivating in hypoxia as compared with fish aestivating in normoxia on day 12 (Table 6.2).

6.3.5. Activity and kinetic properties of hepatic GDH

For fish aestivating in normoxia on day 12, there was a significant increase in the hepatic GDH amination activity, assayed in the presence of saturating concentrations of substrates and ADP, and thus a significant increase in the amination/deamination ratio as compared with fish in preparation for (day 3) or entering into aestivation (day 6) in normoxia (Table 6.3). Similar changes were observed in fish exposed to hypoxia, but they occurred much earlier on day 6 when the dried mucus cocoon was formed. As a result, when assayed in the presence of ADP, the GDH amination activity and amination/deamination ratio from the liver of fish entering into aestivation in hypoxia were significantly greater than those of fish entering into aestivation in normoxia on day 6 (Table 6.3). On day 12, there was a drastic decrease in the hepatic GDH amination activity assayed in the absence of ADP, resulting in a significant smaller amination/deamination ratio, in fish aestivating in normoxia (Table 6.4). It is apparent from these results that the hepatic GDH amination activity became heavily

dependent on ADP activation during the maintenance phase of aestivation in normoxia. Once again, similar changes occurred but much earlier in the hypoxic fish entering into aestivating on day 6 (Table 6.4).

The kinetic properties of an enzyme can be presented as ratios of the enzyme activity assayed at a saturating concentration of substrate ($V_{control}$) versus those assayed at sub-saturating concentrations of substrate. Specifically, an increase and a decrease of the ratio implie a decrease and an increase, respectively, in the affinity of the enzyme to the substrate. Judging by the ratios of the hepatic GDH amination activity assayed at a saturating concentration of α-KG (10 mmol l^{-1}; $V_{control}$) versus those assayed at sub-saturating concentrations of α-KG (0.1, 0.25 or 0.5 mmol l^{-1}), the GDH from the liver of fish entering into aestivation in normoxia on day 6 had a higher apparent affinity towards α-KG as compared with the normoxic fish in preparation for aestivation on day 3 or undergoing aestivation on day 12 (Table 6.5). However, there were no significant differences in the kinetic properties of hepatic GDH in the deamination direction between fish exposed to normoxia on day 3, entering into aestivation on day 6 and undergoing aestivation on day 12 (Table 6.6).

By contrast, the induction and maintenance of aestivation in hypoxia led to a completely different pattern of changes in the kinetic properties of hepatic GDH. On days 6 and 12, the ratios of the hepatic GDH amination activity assayed at a saturating concentration of α-KG (10 mmol l^{-1}; $V_{control}$) versus those assayed at sub-saturating concentrations of α-KG (0.1, 0.25 or 0.5 mmol l^{-1}) obtained from the hypoxic fish were significantly greater than those obtained from the normoxic fish (Table 6.5). These results imply that the apparent affinity of GDH towards α-KG in the normoxic fish was greater than that in the hypoxic fish. In addition, the ratios of the hepatic GDH deamination activity assayed at a saturating concentration of glutamate (100 mmol l^{-1}; $V_{control}$) versus those assayed at sub-saturating concentrations of glutamate (0.5 or 5 mmol l^{-1}) obtained from fish aestivating in hypoxia

were significantly greater than those obtained from fish aestivating in normoxia on day 12 (Table 6.6), indicating an apparent decrease in the affinity towards glutamate in the hypoxic fish as compared with the normoxic fish.

An analysis of the ratios of $V_{control}$ determined in the presence of ADP versus activities determined in the absence of ADP ($V_{minus\ ADP}$) confirmed that the hepatic GDH amination (Table 6.5) and deamination (Table 6.6) activities from *P. annectens* were dependent on ADP activation. Results obtained also confirm that the magnitude of ADP dependency for GDH in the deamination direction remained relatively constant during the 12-day period of exposure to normoxia (Table 6.6). However, a significantly greater dependency on ADP activation was detected for GDH, in the amination direction, extracted from livers of fish aestivating in normoxia on day 12 (Table 6.5) and from livers of fish entering into aestivation on day 6 or maintaining aestivation on day 12 in hypoxia (Table 6.5).

6.3.6. Ammonia and urea excretion rate in fish immersed in water

Rates of ammonia and urea excretion remained relatively constant during 12 days of fasting in water (Fig. 6.4). The average rates of ammonia and urea excretion over the 12-day period were 2.4 ± 0.1 and 0.69 ± 0.05 μmol day^{-1} g^{-1} fish, respectively. Since the tissue urea concentrations were maintained at steady states, the average daily rate of urea synthesis can be taken as 0.69 ± 0.05 μmol day^{-1} g^{-1} fish. Similarly, the average daily rate of endogenous N production (as urea-N + ammonia-N) can be taken as (0.69 x 2) + 2.4 or 3.78 μmol N day^{-1} g^{-1}.

6.3.7. Calculated results for a 100 g fish

Based on the value of 3.78 μmol N day^{-1} g^{-1} (from Fig. 6.4), for a 100 g fish, this would amount to a daily N excretion of 378 μmol. Therefore, a total of 378 μmol day^{-1} x 12

days or 4536 µmol N would have to be accounted for in a 100 g fish, assuming a complete impediment of ammonia and urea excretion.

For a 100 g fish aestivated in normoxia for 12 days, the urea-N accumulated in the muscle (55 g) and the liver (2 g) amounted to 2006 x 2 or 4012 µmol N (from Fig. 6.3), which is approximately 88% of the deficit of 4536 µmol N in nitrogenous excretion. Hence, the rate of urea synthesis in the normoxic fish can be calculated as 2006/(12 days x 100 g) or 1.67 µmol day^{-1} g^{-1}, indicating that it increased 1.67/0.69 or 2.4-fold as compared with the immersed control. Since tissue ammonia concentrations remained unchanged, the rate of endogenous N production (i.e. as ammonia but detoxified to urea) can be calculated as 1.67 x 2 or 3.34 µmol N day^{-1} g^{-1}, which is only 12% lower than the value of 3.78 µmol N day^{-1} g^{-1} for fish immersed in water.

By contrast, only 945 µmol of excess urea was accumulated in muscle and liver of a 100 g fish in hypoxia on day 12, which (945 x 2 = 1890 µmol) represents approximately 42% of the deficit of 4536 µmol N in nitrogenous excretion. Hence, the estimated average urea synthesis rate during the 12-day period is 945/(12 days x 100 g) or 0.79 µmol day^{-1} g^{-1}, which implies that the average rate of urea synthesis in the hypoxic fish was comparable to (1.1-fold) that (0.69 µmol day^{-1} g^{-1}) of fish immersed in water. The average rate of endogenous N production can be calculated as 1890 µmol/(100 g x 12 days) or 1.58 µmol day^{-1} g^{-1}, which represents a decrease by 58% below the rate in fish immersed in water for 12 days (3.78 µmol day^{-1} g^{-1}), and such a decrease is much greater than that (12%) observed in fish undergoing induction and maintenance of aestivation in normoxia.

Table 6.1. Concentrations (μmol g^{-1} wet mass or μmol ml^{-1} plasma) of ammonia in the muscle, liver and plasma of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

Tissue	Normoxia			Hypoxia		
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
Muscle	0.27 ± 0.10	0.16 ± 0.05	0.15 ± 0.06	0.22 ± 0.23	0.71 ± 0.60	0.37 ± 0.15
Liver	1.35 ± 0.36	0.84 ± 0.19	0.47 ± 0.06	2.45 ± 1.07	2.07 ± 1.13	0.91 ± 0.22
Plasma	0.51 ± 0.06	0.49 ± 0.05	0.37 ± 0.04	0.67 ± 0.14	0.45 ± 0.11	0.47 ± 0.06

Results are presented as means ± S.E.M. (*N*=5 for control and *N*=4 for hypoxia).

Table 6.2. Concentrations (μmol g^{-1} wet mass) of various free amino acids (FAAs) that showed significant changes, total essential FAA (TEFAA) and total FAA (TFAA) in the muscle and liver of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

Tissue	FAA	Normoxia			Hypoxia		
		Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
Muscle	Arginine	0.0079 ± 0.0051	0.024 ± 0.003	0.026 ± 0.001	0.030 ± 0.002*	0.021 ± 0.002	0.023 ± 0.003
	Leucine	0.217 ± 0.0134	0.171 ± 0.043	0.195 ± 0.019	0.323 ± 0.045*	0.252 ± 0.028	0.157 ± 0.022
	Phenylalanine	0.076 ± 0.003	0.057 ± 0.017	0.040 ± 0.003	0.114 ± 0.013*	0.082 ± 0.010	0.058 ± 0.007
	Tyrosine	0.162 ± 0.012	0.208 ± 0.025	0.189 ± 0.020	0.223 ± 0.017*	0.305 ± 0.035*	0.252 ± 0.050
	TEFAA	2.20 ± 0.41	2.07 ± 0.41	1.57 ± 0.19	3.16 ± 0.25	2.79 ± 0.50	2.40 ± 0.43
	TFAA	3.97 ± 0.43	3.77 ± 0.70	3.52 ± 0.48	5.02 ± 0.42	5.53 ± 0.88	4.53 ± 0.94
Liver	Alanine	0.186 ± 0.037	0.065 ± 0.026	0.095 ± 0.033	0.265 ± 0.060	0.491 ± 0.044*	0.108 ± 0.013
	Glutamate	1.61 ± 0.307	1.30 ± 0.30	1.01 ± 0.24	2.64 ± 0.48	4.34 ± 0.227*	1.92± 0.15*
	Proline	0.140 ± 0.069	0.101 ± 0.014	0.138 ± 0.049	0.568 ± 0.277	0.298 ± 0.071*	0.110 ± 0.016
	Tryptophan	0.481 ± 0.302	1.44 ± 0.45	N.D.	1.16 ± 0.284	0.820 ± 0.235	0.509 ± 0.119*
	Tyrosine	0.104 ± 0.018	0.238 ± 0.039	0.144 ± 0.015	0.183 ± 0.018*	0.247 ± 0.052	0.167 ± 0.033
	TEFAA	2.13 ± 0.43	2.89 ± 0.55	1.13 ± 0.12	3.52 ± 0.36*	2.96 ± 0.80	1.85 ± 0.15*
	TFAA	5.64 ± 1.00	6.77 ± 0.78	4.47 ± 0.68	8.99 ± 0.68*	10.28 ± 0.44*	5.57 ± 0.31

Results represent means ± S.E.M. (N=4)

*Significantly different from the corresponding normoxic value (p<0.05).

Table 6.3. Specific activities of glutamate dehydrogenase (GDH) in the amination (μmol NADH oxidized min^{-1} g^{-1} wet mass) and deamination (μmol formazan formed min^{-1} g^{-1} wet mass) directions assayed at saturating concentrations of substrates (10 $mmol^{-1}$ α-ketoglutarate and 100 mmol l^{-1} glutamate, respectively) in the presence of 1 mmol l^{-1} ADP ($V_{control}$), and their ratios (amination/deamination) from the liver of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

GDH	Normoxia			Hypoxia		
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
Amination $V_{control}$	16.9 ± 0.9[a]	18.6 ± 2.7[a]	32.1 ± 4.4[b]	19.2 ± 1.1[a]	28.3 ± 1.4[b *]	32.9 ± 2.0[b]
Deamination $V_{control}$	0.92 ± 0.03	1.51 ± 0.33	0.91 ± 0.04	0.87 ± 0.03	0.90 ± 0.08	1.07 ± 0.12
Amination/deamination	18.0 ± 1.0[a]	13.3 ± 1.6[a]	34.5 ± 3.4[b]	22.0 ± 2.0[a]	31.2 ± 1.1[b *]	31.6 ± 1.8[b]

Results represent means ± S.E.M. (*N*=5)

Means not sharing the same letter are significantly different ($p<0.05$).

[*]Significantly different from the corresponding normoxic value ($p<0.05$).

Table 6.4. Specific activities of glutamate dehydrogenase (GDH) in the amination (μmol NADH oxidized min^{-1} g^{-1} wet mass) and deamination (μmol formazan formed min^{-1} g^{-1} wet mass) directions assayed at saturating concentrations of substrates (10 $mmol^{-1}$ α-ketoglutarate and 100 mmol l^{-1} glutamate, respectively) in the absence of ADP ($V_{minus\ ADP}$), and their ratios (amination/deamination) from the liver of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

GDH	Normoxia			Hypoxia		
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
Amination $V_{minus\ ADP}$	2.20 ± 0.39 [b]	3.82 ± 0.6 [c]	0.12 ± 0.06 [a]	3.04 ± 0.25 [b]	0.16 ± 0.09 [a] *	0.16 ± 0.07 [a]
Deamination $V_{minus\ ADP}$	0.17 ± 0.01	0.26 ± 0.04	0.24 ± 0.03	0.16 ± 0.02	0.16 ± 0.02	0.19 ± 0.01
Amination/deamination	13.6 ± 2.8 [b]	15.0 ± 0.5 [b]	0.53 ± 0.22 [a]	18.9 ± 0.8 [b]	0.93 ± 0.52 [a] *	0.91 ± 0.39 [a]

Results represent means ± S.E.M. (*N*=5)

Means not sharing the same letter are significantly different ($p<0.05$).

*Significantly different from the corresponding normoxic value ($p<0.05$).

Table 6.5. Ratios of activities of glutamate dehydrogenase in the amination direction assayed in the presence of 1 mmol l^{-1} ADP at saturating (10 mmol l^{-1}, control) versus sub-saturating (0.5, 0.25 or 0.1 mmol l^{-1}) concentrations of α-ketoglutarate (α-KG), and ratios of enzyme activities assayed at 10 mmol l^{-1} α-KG in the presence of ADP (1 mmol l^{-1}, control) versus in the absence of ADP from the liver of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

GDH, amination	Normoxia			Hypoxia		
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
$V_{control}/V_{0.5\ mM\ \alpha KG}$	1.32 ± 0.02 ^{b}	1.18 ± 0.03 ^{a}	1.33 ± 0.02 ^{b}	1.29 ± 0.05 ^{a}	1.41 ± 0.03 ^{ab} *	1.45 ± 0.01 ^{b} *
$V_{control}/V_{0.25\ mM\ \alpha KG}$	2.06 ± 0.04 ^{b}	1.84 ± 0.06 ^{a}	2.18 ± 0.06 ^{b}	2.05 ± 0.07 ^{a}	2.34 ± 0.06 ^{b} *	2.41 ± 0.02 ^{b} *
$V_{control}/V_{0.1\ mM\ \alpha KG}$	4.40 ± 0.16 ^{b}	3.89 ± 0.11 ^{a}	4.81 ± 0.09 ^{b}	4.72 ± 0.13	5.12 ± 0.12 *	5.15 ± 0.18
$V_{control}/V_{minus\ ADP}$	8.82 ± 1.88 ^{a}	4.88 ± 0.14 ^{a}	110 ± 32 ^{b}	6.38 ± 0.26 ^{a}	55 ± 16 (4) ^{ab} *	190 ± 74 ^{b}

Results represent means ± S.E.M. (*N*=5)

Means not sharing the same letter are significantly different ($p<0.05$).

*Significantly different from the corresponding normoxic value ($p<0.05$).

Table 6.6. Ratios of activities of glutamate dehydrogenase in the deamination direction assayed in the presence of 1 mmol l^{-1} ADP at saturating (100 mmol l^{-1}, control) versus sub-saturating (5 or 0.5 mmol l^{-1}) concentrations of glutamate (Glu), and ratios of enzyme activities assayed at 100 mmol l^{-1} Glu in the presence of ADP (1 mmol l^{-1}, control) versus the absence of ADP from the liver of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia or hypoxia (2% O_2 in N_2)

GDH, deamination	Normoxia			Hypoxia		
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
$V_{control}/V_{5\ mM\ Glu}$	1.16 ± 0.02	1.14 ± 0.03	1.06 ± 0.03	1.16 ± 0.06	1.10 ± 0.02	1.17 ± 0.02 *
$V_{control}/V_{0.5\ mM\ Glu}$	9.89 ± 1.54	7.92 ± 0.63	5.96 ± 1.85	11.0 ± 1.9	15.6 ± 5.6	13.8 ± 2.6 *
$V_{control}/V_{minus\ ADP}$	5.58 ± 0.43	5.93 ±0.92	4.05 ± 0.52	5.66 ± 0.86	5.69 ± 0.32	5.61 ± 0.59

Results represent means ± S.E.M. (N=5)

*Significantly different from the corresponding normoxic value (p<0.05).

Fig. 6.1. Concentrations (μmol g^{-1} wet mass) of adenosine triphosphate (ATP), as determined by *in vivo* ^{31}P NMR spectroscopy, in the (A) anterior, (B) middle and (C) posterior regions of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia (open circle) or hypoxia (2% O_2 in N_2; closed circle) as compared with the day 0 value (in water). Values are means ± S.E.M. (*N*=3 for normoxia, *N*=4 for hypoxia). *Significantly different from the corresponding normoxia value in that region of the body on that day ($p<0.05$).

Fig. 6.1.

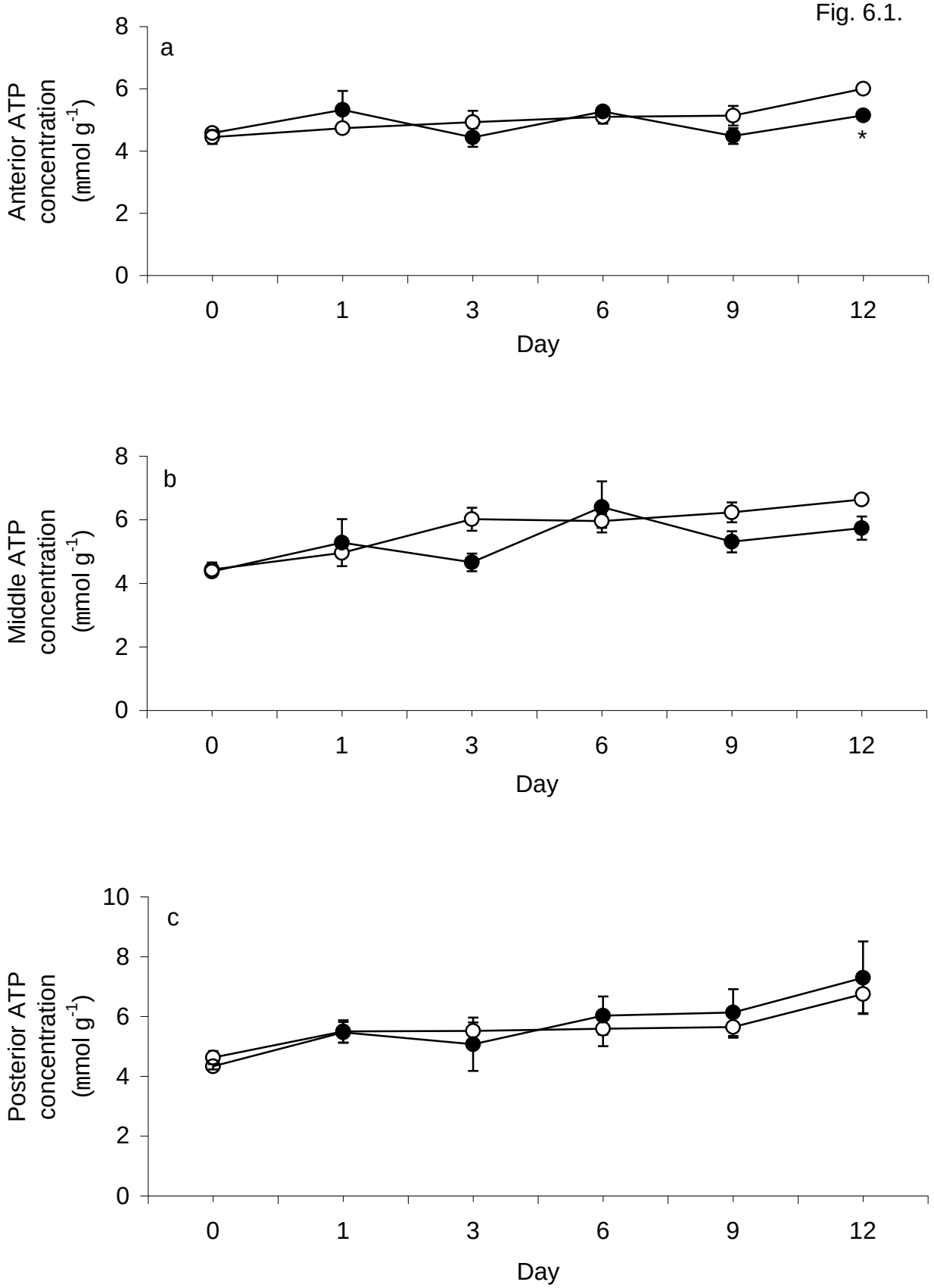

a
Anterior ATP concentration (mmol g-1)
8
6
4
2
0
0
1
3
6
9
12
Day
*
b
Middle ATP concentration (mmol g-1)
8
6
4
2
0
0
1
3
6
9
12
Day
c
Posterior ATP concentration (mmol g-1)
10
8
6
4
2
0
0
1
3
6
9
12
Day

Fig. 6.2. Concentrations (μmol g^{-1} wet mass) of creatine phosphate, as determined by *in vivo* ^{31}P NMR spectroscopy, in the (a) anterior, (b) middle and (c) posterior regions of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia (open circle) or hypoxia (2% O_2 in N_2; closed circle) as compared with the day 0 value (in water). Values are means ± S.E.M. (*N*=3 for normoxia, *N*=4 for hypoxia). *Significantly different from the corresponding normoxia value in that region of the body on that day ($p<0.05$).

Fig.6.2.

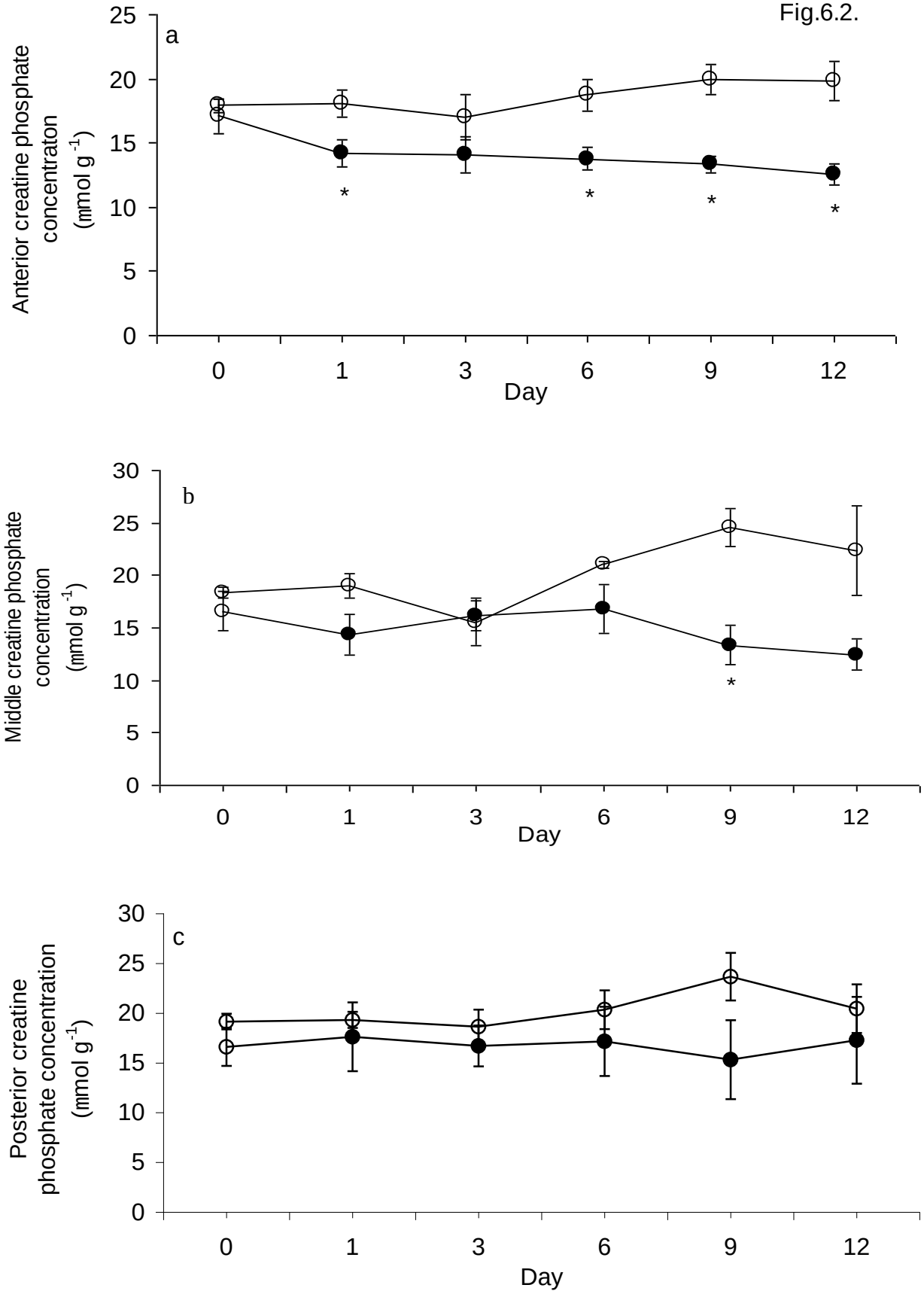

a
Anterior creatine phosphate concentraton (mmol g-1)
25
20
15
10
5
0
0
1
3
6
9
12
Day
*
*
*
*
b
Middle creatine phosphate concentration (mmol g-1)
30
25
20
15
10
5
0
0
1
3
6
9
12
Day
*
c
Posterior creatine phosphate concentration (mmol g-1)
30
25
20
15
10
5
0
0
1
3
6
9
12
Day

Fig. 6.3. Concentrations (μmol g^{-1} wet mass or μmol ml^{-1} plasma) of urea in (a) muscle, (b) liver and (c) plasma of *Protopterus annectens* during 12 days of induction and maintenance of aestivation in normoxia (open bar) or hypoxia (2% O_2 in N_2; closed bar). Values are means + S.E.M. (*N*=5 for control and *N*=4 for hypoxia). Means not sharing the same letter are significantly different ($p<0.05$). *Significantly different from the corresponding normoxic value ($p< 0.05$).

Fig. 6.3.

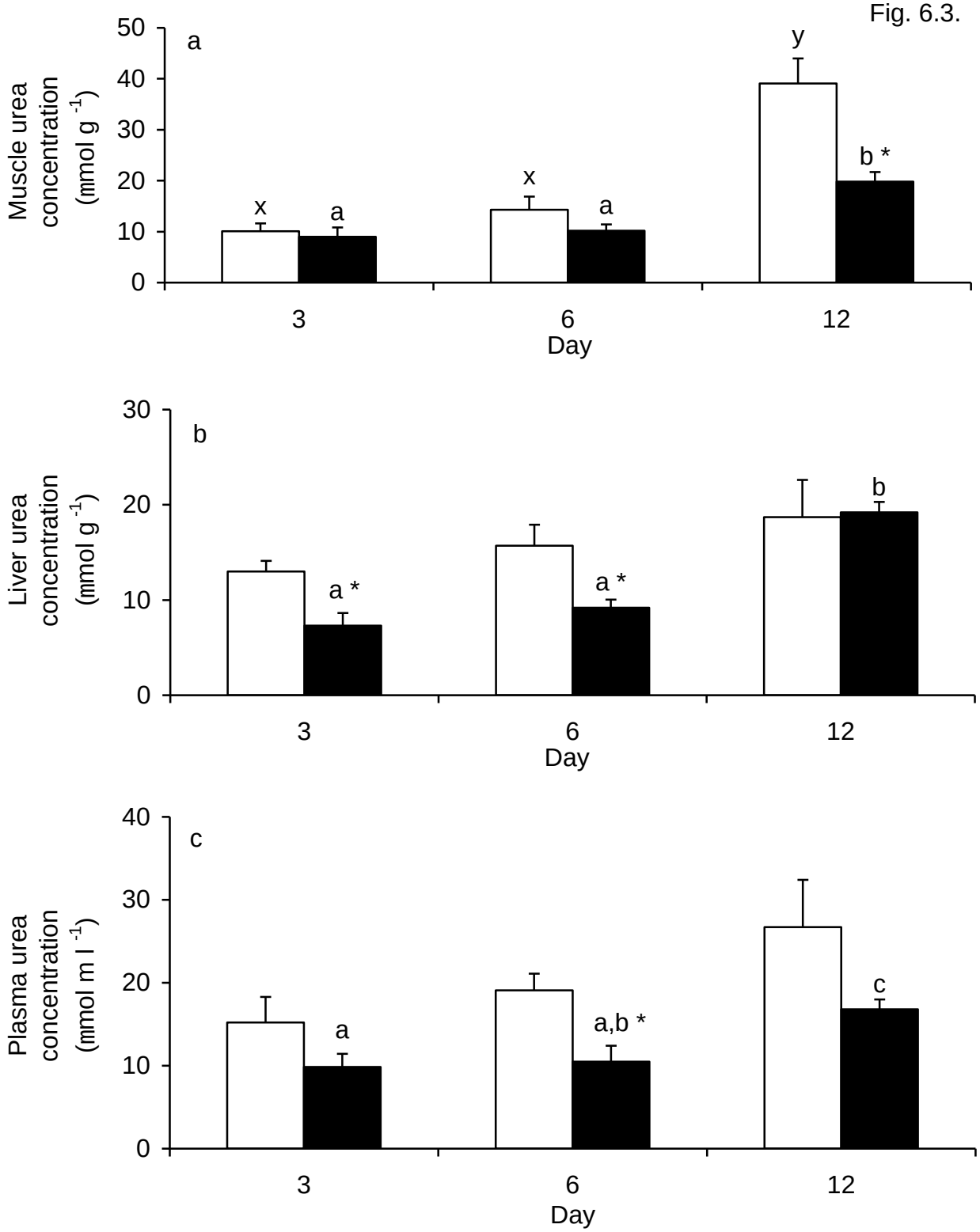
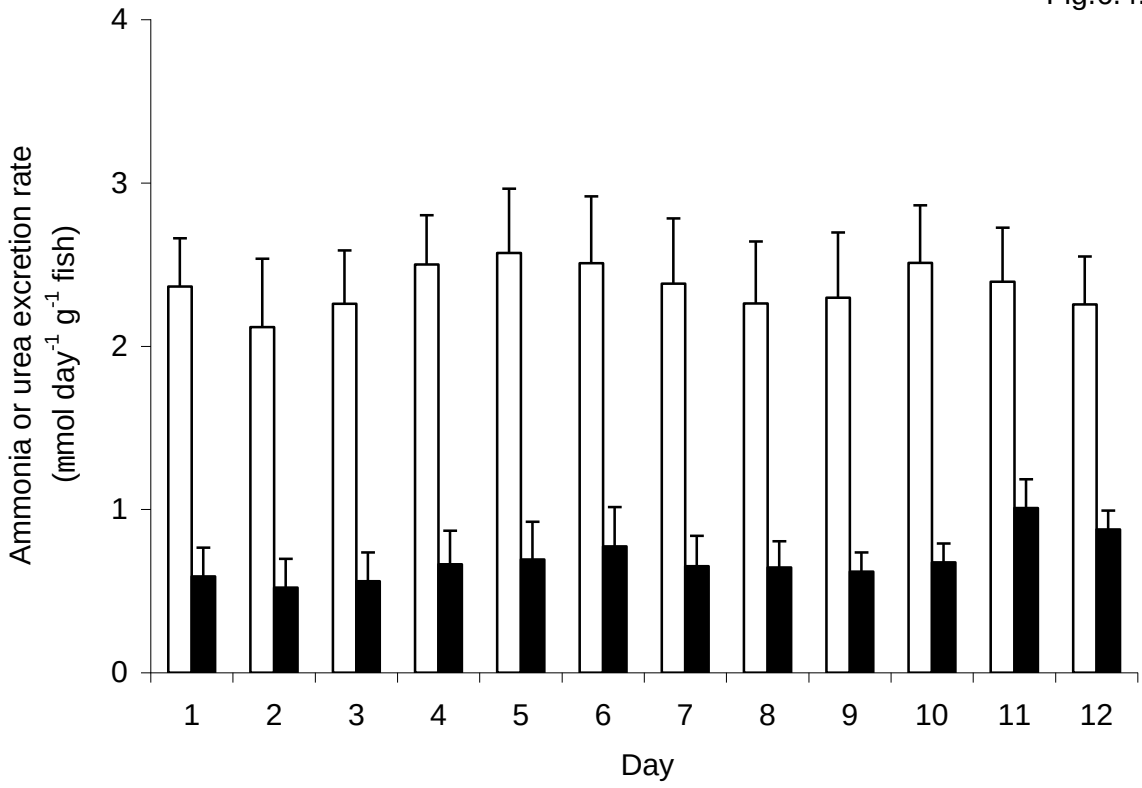

a
Muscle urea concentration (mmol g -1)
50
40
30
20
10
0
x
a
x
a
y
b *
3
6
12
Day
b
Liver urea concentration (mmol g -1)
30
20
10
0
a *
a *
b
3
6
12
Day
c
Plasma urea concentration (mmol m l -1)
40
30
20
10
0
a
a,b *
c
3
6
12
Day

Fig. 6.4. Rates (μmol day^{-1} g^{-1} fish) of ammonia (open bar) and urea (closed bar) excretion in *Protopterus annectens* during 12 days of fasting in water. Values are means + S.E.M. (*N*=5).

Fig.6.4.


Ammonia or urea excretion rate
(mmol day-1 g-1 fish)
0
1
2
3
4
1
2
3
4
5
6
7
8
9
10
11
12
Day

6.4. Discussion

6.4.1. Hypoxia led to lower ATP and creatine phosphate concentrations in certain body regions in comparison with normoxia at certain time point

Based on results obtained from *in vivo* ^{31}P NMR spectroscopy (Fig. 6.1 and 6.2), it can be concluded that, in general, hypoxia led to lower concentrations of ATP and creatine phosphate in *P. annectens* during 12 days of aestivation as compared with normoxia. These results are novel and suggest that information available in the literature on African lungfishes aestivating in mud or an artificial device/substratum should be interpreted with caution (as suggested by Loong et al., 2008b), because those information cannot be interpreted simply as effects of aestivation alone (Storey, 2002), and they may actually reflect the combined effects of aestivation and hypoxia.

6.4.2. Induction and maintenance of aestivation in normoxia or hypoxia did not affect tissue ammonia concentrations but hypoxia led to a much smaller accumulation of urea

Although it has been reported previously that African lungfishes do not accumulate ammonia during aestivation because of increased urea synthesis and/or decreased endogenous ammonia production (Chew et al., 2004; Ip et al., 2005f; Loong et al., 2008b), results from this study indicate for the first time that the magnitude of changes in urea synthesis and ammonia production in fish aestivating in hypoxia differed from those in normoxia. For fish undergoing 12 days of aestivation in normoxia, there was a 2.4-fold increase in the rate of urea synthesis, but the rate of ammonia production decreased by only 12%, as compared with the immersed control. By contrast, the average rate of urea synthesis remained relatively unchanged (1.1-fold), but there was a prominent (58%) decrease in ammonia production, in fish aestivating in hypoxia. In normoxia, the energy status remained relatively high throughout the 12-day period, and *P. annectens* was able to depend mainly on increased urea synthesis, which is an energy-intensive process, to avoid ammonia toxicity. However, in

hypoxia where conservation of cellular energy became an important issue, it avoided ammonia toxicity mainly through reduced ammonia production.

It has been suggested previously that aestivation in air entails desiccation, and that increased tissue urea concentrations might serve the secondary function of facilitating water retention in tissues through vapour pressure depression (Campbell, 1973; Loong et al., 2008). In this study, the two groups of experimental fish underwent aestivation in a closed box with similar flow rates of air or 2% O_2 in N_2, which ensured similar rates of desiccation, but fish aestivating in hypoxia exhibited a greater magnitude of reduction in ammonia production and accumulated much less urea. Therefore, our results indicate for the first time that increased urea synthesis in *P. annectens* (and probably other African lungfishes) during aestivation is an adaptation responding primarily to ammonia toxicity, and that the involvement of urea in reducing evaporative water loss could be a secondary phenomenon dependent on the availability of sufficient oxygen.

6.4.3. Aestivation in hypoxia resulted in changes in tissue FAA concentrations

The steady-state concentrations of tissue amino acids are maintained by a balance between the rates of their degradation and production. Alteration in this balance would lead to shifts in concentrations. For fish used in this study, amino acids would be produced mainly through protein degradation because food was withdrawn 96 h prior to and during the experiments. Since there was a significant increase in the TFAA concentration in the liver of *P. annectens* in hypoxia on days 3 and 6 as compared with that of the normoxic fish, it is logical to assume that a reduction in amino acid catabolism had occurred, resulting in the accumulation of FAAs and hence an increase in the TFAA concentration. In addition, there was a significant increase in the TEFAA concentration in the liver of fish exposed (on day 3) to or aestivating (on day 12) in hypoxia as compared with those of fish in normoxia. Since essential amino acids could not be synthesized by the fish and since there was no food

supply, they must have been released through protein degradation. Therefore, increases in their concentrations could be a result of an increase in protein degradation or a decrease in their catabolism. The latter seems to be a more probable proposition than the former because of the needs to avoid ammonia toxicity during aestivation in the absence of water.

Incidentally, there was a significant increase in the glutamate concentration in the liver of fish aestivating in hypoxia on days 6 and 12. Glutamate is a key amino acid involved in the synthesis of many non-essential amino acids through various transamination reactions. In addition, it acts as the substrate and the product for the GDH deamination and GDH amination reactions, respectively. The increase in glutamate concentration in tissues of the hypoxic fish suggests an alteration in the rates of production and/or degradation of glutamate, and it may also indicate a reduction in glutamate transdeamination which would reduce ammonia production. Overall, these results indicate that there was a concerted effort in *P. annectens* to minimize energy expenditure in relation to ammonia detoxification during aestivation in hypoxia.

6.4.4. Activities and properties of hepatic GDH from the liver of fish during the induction and maintenance phases of aestivation: normoxia versus hypoxia

For fish exposed to normoxia, the activities of hepatic GDH, in the amination and deamination directions, remained relatively constant during the induction phase (3 or 6 days) of aestivation. However, there was a significant increase in the GDH amination activity, with the deamination activity remained unchanged, in fish aestivating in normoxia on day 12. Hence, GDH would act less favourably in the deamination direction during the maintenance phase of aestivation to reduce the production of ammonia through transdeamination. At the same time, the hepatic GDH amination activity, but not the deamination activity, from fish aestivating in normoxia on day 12 became highly dependent on the presence of ADP. These results indicate that transdeamination of amino acids through the hepatic GDH became

responsive mainly to the cellular energy status of the fish during the maintenance phase of aestivation (day 12) in normoxia.

It has been demonstrated that hepatic GDH activity increases with increased plasma ammonia concentration in juvenile turbot and seabream exposed to environmental ammonia (Person Le Ruyet et al., 1998). However, the ammonia concentrations in various tissues of *P. annectens* exposed to normoxia (or hypoxia) remained relatively unchanged and thus it can be concluded that changes in the activity of hepatic GDH occurred primarily to reduce ammonia production, and not to detoxify ammonia during aestivation. More importantly, results from this study reveal that changes could occur in the amination activity of GDH without any change in its deamination activity. Hence, a cautious approach should be taken to interpret results on GDH in the literature, which involved only the determination of amination activity but with the assumption that similar changes would occur in the deamination direction.

For fish exposed to hypoxia, significant increases in the hepatic GDH amination activity, the amination/deamination ratio and the dependency of the amination activity on ADP activation occurred much earlier on day 6, i.e. at the onset of aestivation, instead of day 12. These results indicate that, decreased ammonia production through changes in the activity of hepatic GDH in *P. annectens* can be more effectively induced and exacerbated by a combination of aestivation and hypoxia then aestivation alone (in normoxia). To my knowledge, this is the first report of such a phenomenon in African lungfishes. These results indicate that GDH was critically regulated in fish during the induction phase of aestivation in hypoxia, suppressing ammonia production in order to reduce the dependency on increased urea synthesis to detoxify ammonia. From these results, it can be deduced that *P. annectens* could aestivate for a longer period in hypoxia than in normoxia by conserving cellular energy through decreased ammonia production and urea synthesis and slowing down amino acid catabolism through changes in GDH activity.

There was apparently no change in the affinity of the hepatic GDH to glutamate in the deamination direction during 12 days of aestivation in normoxia. However, there was an apparent increase in the affinity of the GDH to α–KG in the amination direction, which occurred only transiently on day 6 when the fish entered into aestivation in normoxia. This change in kinetic property can theoretically lead to an increase in the amination/deamination ratio at low concentration of α–KG and result in less ammonia being produced through transdeamination. By contrast, a close examination of the kinetic properties of GDH from hypoxic fish reveals that there was an apparent decrease in the affinity to α–KG in the amination direction on day 6 and 12 in hypoxia, and it occurred in spite of an increase in the $V_{control}$. There is also decrease in affinity of GDH of hypoxic fish to glutamate. Although, there is a 2-fold increase in glutamate in hypoxic fish than normoxic fish, due to compartmentalization of GDH in mitochondria, the increase in glutamate, if occur in cytosol, will not be able to compensate for the affinity of GDH to substrate. Overall, there is a decrease in activity of GDH in hypoxic fish and the increase in glutamate concentration could be a result of the decrease in the deaminating being greater than the decrease in aminating activity.

In general, GDH can be regulated by ADP-ribosylation, and Herrero-Yraola et al. (2001) showed that modification and concomitant inhibition of GDH were reversed enzymatically by an ADP-ribosylcysteine hydrolase *in vivo*. It is also known that two GDH isoforms (GLUD1 and GLUD2) exist in *Homo sapiens* (Plaitakis and Zaganas, 2001). Additionally, there are two distinct forms of GDH with different affinities for glutamate, ammonia and α–ketoglutarate in Richardson's ground squirrel, *S. richardsonii*, and entry into hibernation leads to changes in the properties of GDH that enables it to function optimally to suit the environment (Thatcher and Storey, 2001). Hence, the possibility that different forms of GDH were expressed in *P. annectens* during aestivation, specifically during entering into aestivation on day 6 (for fish aestivating in hypoxia) and undergoing aestivation on day 12

(for fish aestivating in normoxia), cannot be ignored. Taken together, these results support the proposition that hypoxia could have induced the expression of GDH isoforms or the post-transcriptional modification of GDH in the liver of *P. annectens* much earlier than normoxia in preparation for aestivation.

6.4.5. Conclusion

Results from this study indicate for the first time that *P. annectens* exhibited different adaptive responses during the induction and maintenance phases of aestivation in normoxia and in hypoxia. It avoided ammonia toxicity mainly through increased urea synthesis and reduced ammonia production during 12 days of aestivation in normoxia and hypoxia, respectively. Hypoxia resulted in changes in activities of hepatic GDH, in the amination direction, on days 6 and 12, but similar changes occurred in the normoxic fish on day 12 only. Hence, reduction in nitrogen metabolism, and possibly in metabolic rate, occurred more prominently in response to a combined effect of aestivation and hypoxia, and a re-examination of the intricate relationships between aestivation, hypoxia and metabolic rate reduction in African lungfishes is warranted. Additionally, these results suggest that information available in the literature concerning aestivating lungfishes should be viewed with caution, especially when no indication was provided on whether aestivation occurred in normoxia or hypoxia as in the case of aestivation in air or in mud or artificial substratum, or on the severity of hypoxia that was involved.

6.5. Summary

The author examined the energy status, nitrogen metabolism and hepatic glutamate dehydrogenase activity in the African lungfish *Protopterus annectens* during aestivation in normoxia (air) or hypoxia (2% O_2 in N_2), with tissues sampled on day 3 (aerial exposure with preparation for aestivation), day 6 (entering into aestivation) or day 12 (undergoing aestivation). There was no accumulation of ammonia in tissues of fish exposed to normoxia or hypoxia throughout the 12-day period. Ammonia toxicity was avoided by increased urea synthesis and/or decreased endogenous ammonia production, but the dependency on these two mechanisms differed between the normoxic and the hypoxic fish. The rate of urea synthesis increased 2.4-fold, with only a 12% decrease in the rate of ammonia production in the normoxic fish. By contrast, the rate of ammonia production in the hypoxic fish decreased by 58%, with no increase in the rate of urea synthesis. Using *in vivo* ^{31}P NMR spectroscopy, it was demonstrated that hypoxia led to significantly lower ATP concentration on day 12 and significantly lower creatine phosphate concentration on days 1, 6, 9 and 12 in the anterior region of the fish as compared with normoxia. Additionally, the hypoxic fish had lower creatine phosphate concentration in the middle region than the normoxic fish on day 9. Hence, lowering the dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, by reducing ammonia production, would conserve cellular energy during aestivation in hypoxia. Indeed, there were significant increases in glutamate concentrations in tissues of fish aestivating in hypoxia, which indicates decreases in its degradation and/or transamination. Furthermore, there were significant increases in the hepatic glutamate dehydrogenase amination activity, the amination/deamination ratio and the dependency of the amination activity on ADP activation in fish on days 6 and 12 in hypoxia, but similar changes occurred only in the normoxic fish on day 12. Therefore, these results indicate for the first time that *P. annectens* exhibited different adaptive responses during aestivation in normoxia and in hypoxia. They also indicate that reduction in nitrogen metabolism, and probably

metabolic rate, did not occur simply in association with aestivation (in normoxia) but responded more effectively to a combined effect of aestivation and hypoxia.

7. Chapter 4:

Using suppression subtractive hybridization PCR to evaluate up- and down-expression of gene clusters in the liver of *Protopterus annectens* during the onset of aestivation (day 6) in normoxia or hypoxia (2% O_2 in N_2)

7.1. Introduction

Protopterus annectens is one of the four species of African lungfishes that can undergo aestivation in the absence of water during drought, and remain incarcerated in this state of inactivity until the return of water to the habitat (Fishman et al., 1987; Ip et al., 2005a). It can aestivate inside a cocoon made of dried mucus in air or burrow into the mud and aestivate in a subterranean cocoon (Chapter 2; Loong et al., 2008b). Despite being ammonotelic in water, *P. annectens* is ureogenic and possesses a full complement of ornithine-urea cycle (OUC) enzymes, including carbamoyl phosphate synthetase III (CPS III) which require glutamine as a substrate, in the liver (Chapter 1; Loong et al., 2005). During aestivation, ammonia excretion would be impeded, leading to its accumulation in the body. Since ammonia is toxic (Cooper and Plum, 1987; Hermenegildo et al., 1996; Ip et al., 2001; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002), *P. annectens* has to avoid ammonia toxicity during aestivation, and results obtained by the author indicate that they achieve this through an increase in urea synthesis and a suppression of ammonia production (Chapter 2; Loong et al., 2008b).

Loong et al. (2008b) reported that *P. annectens* that underwent 46 days of aestivation (i.e. 6 days of induction phase plus 40 days of maintenance phase) in mud had a lower dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, than fish aestivating in air (Chapter 2). Subsequently, Loong et al. (2008a) demonstrated that *P. annectens* exhibited different adaptive responses during 12 days of aestivation (6 days of induction phase plus 6 days of maintenance phase) in normoxia and in hypoxia (Chapter 3); their results indirectly support the proposition that reduction in nitrogen metabolism, and probably metabolic rate, did not occur simply in association with aestivation (in normoxia) but responded more effectively to a combined effect of aestivation and hypoxia. In order to confirm that indeed aestivating *P. annectens* responds differently to normoxia and hypoxia (2% O_2 in N_2), this study was undertaken to compare and contrast the effects of 6 days of

aestivation in normoxia and 6 days of aestivation in hypoxia on up- and down-regulations of gene expressions in the liver of *P. annectens*, using suppression subtractive hybridization (SSH) PCR.

Why would the author decide on adopting the SSH approach in this study? Other than SSH, there are a variety of available tools that allows the identification of differentially expressed transcripts between two populations of mRNA. These include differential analysis of library expression (DAZLE) (Li et al., 2004), differential display (Liang et al., 1992; Sokolov et al., 1994), representational difference analysis (Lisitsyn et al., 1993), enzymatic degradation subtraction (Zeng et al., 1994), linker capture subtraction (Yang et al., 1996), techniques involving physical removal of common sequences (Akopian et al., 1995; Deleersnijder et al., 1996) and serial analysis of gene expression (Velculescu et al., 1995). Despite the fact that these methods have proven successful in isolation of differentially expressed genes, they all possess some intrinsic drawbacks. In addition to their specific limitations, a common feature is the inability to isolate rare transcripts, i.e., the disproportion of concentrations of differentially expressed genes is maintained in the subtraction (Munir et al., 2004). By contrast, SSH generates an equalized representation of differentially expressed genes irrespective of their relative abundance (Diatchenko et al., 1996).

At present, SSH is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries (Lukyanov et al., 1994; Diatchenko et al., 1996; Gurskaya et al., 1996; Jin et al., 1997; Akopyants et al., 1998). SSH method is based on a suppression PCR effect, introduced by Sergey Lukyanov (Lukyanov et al., 1994). The principle behind SSH is that it is used to look at genes that were up- or down-regulated following a treatment, then the cDNA generated from the mRNA obtained after that treatment will be divided into two equal pools. These pools are known as the “tester”, which are later ligated with two different adapters (adapter 1 and adapter 2). The cDNA from the mRNA obtained from untreated tissues are the “drivers”, which are not ligated. The SSH

method uses two hybridization. First, an excess of driver is added to each sample of the tester. The samples are then heat-denatured and allowed to anneal. This hybridization removes transcripts in the tester that are found in the driver. The second hybridization is performed by adding both testers together with new driver. The addition of a second portion of denatured driver further enriches differentially expressed genes. After filling in the overhanging ends, this is subjected to a first PCR reaction and then a second PCR reaction using nested primer sites within each adaptor. Finally, only DNA that has both of the adaptors will be amplified by PCR in the nested reaction.

The key feature of the method is simultaneous normalization and subtraction steps. The normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the two populations being compared (Gurskaya et al., 1996). SSH eliminates any intermediate steps demanding the physical separation of single-stranded (ss) and double-stranded (ds) DNAs, it requires only one round of subtractive hybridization, and it can achieve a >1000-fold enrichment for differentially presented DNA fragments.

Using SSH, a number of studies had discovered novel genes. Shin et al. (2001) discovered natural killer enhancing factor (NKEF) from the common carp (*Cyprinus carpio*) that received intraperitoneal injection of alginate or scleroglucan using SSH. Prior to that, little is known about the structure and function of NKEF in lower vertebrates. SSH is also used as a technique to clone out osteopontin-like gene in *Sparus aurata* (gilthead seabream). SSH has allowed further study to lead to better understanding on the mechanisms of tissue mineralization and unravelling specific questions related to vertebrate bone formation (Fonseca et al., 2007). Using SSH, Tsuitsui et al. (2007) was able to identifying a conger eel (*Conger myriaster*) interleukin-1β (IL-1β). This is the first report on cytokines in Anguilliformes.

Other than discovering novel genes, SSH is used to prepare libraries representing genes that are differentially expressed in tissues of control animal and treated animals. Bayne et al. (2001) utilized SSH as a method to create cDNA library from the livers of resting rainbow trout and of trout in the course of an acute phase response. The resulting cDNA library contains 300-600 bp long fragments of 25 or more immune-relevant genes. Similarly, Monens et al. (2007) discovered a total of 398 different gene fragments that were most related to endocrine functioning using SSH in *Cyprinus carpio*.

SSH is also gaining popularity as a method use to elicit molecular mechanism of treatment in fish. *Carassius* species have been shown to be highly tolerant of hypoxia. However the molecular basis of the *Carassius* species response to hypoxia has not been clarified. Zhong et al. (2009) utilized SSH to reveal the changes in gene expression in *C. auratus* blastulae embryonic (CAB) cells responding to hypoxia stress. Another study also utilized SSH as a tool to identify novel genomic responses of a commercial agriculture species, *Solea senegalensis*, when injected with lipopolysaccharide and copper sulphate (Prieto-Álamo et al., 2009). In this study, SSH identified a total of 156 genes involved in major physiological functions.

Despite the usefulness of SSH, there are several drawbacks on this method. Firstly, in order to obtain maximum data from cDNA or genomic DNA subtraction experiment, it is important to achieve the highest efficiency of subtraction. The power of SSH subtraction makes it possible to achieve a level of 90-95% differentially expressed clones in the cDNA-subtracted library (Diatchenko et al., 1996; Zuber et al., 2000). In cases where differentially expressed clones represent the majority of the clones in the subtracted library, the time consuming process of differential screening can be omitted. Whenever possible, the researcher should consider designing the experiment to yield the higher level of difference between the tester and driver RNA populations, possibly by choosing the time point with the

higher fold induction of control gene (Rebrikov, 1995). The second drawback of SSH is gene redundancy. If there are a few genes that are highly differentially expressed, then they will appear in the library a large number of times and may mask the isolation of transcripts that are still differentially expressed but at a lower overall level (Goetz, 2003). It is necessary to analyze 500-1000 clones from a subtracted library to ensure that genes representing low-abundance transcripts are not lost (Rebrikov, 1995). Third drawback of SSH is whether all of the genes are truly regulated between the two samples. Goetz (2003) found that even with the subtraction process, there were still a number of false positives in the PCR mixture at the end. For the later step, it is important to have some verification/sorting of the subtracted DNA.

Since SSH involves intensive gene cloning and sequencing, the author decided to focus on the liver of *P. annectens* undergoing 6 days of aestivation in normoxia or hypoxia in this study. The rational behind this decision was that the liver happened to be the major organ involved in amino acid catabolism and in urea synthesis through the OUC. Furthermore, a 6-day period was chosen because it was on day 6 that the external medium dried up with the formation of a mucus cocoon that completely encased the fish. The author aimed to confirm through SSH that mRNA expression of OUC enzymes, especially CPS III, were up-regulated during the transition between the induction phase and the initial maintenance phases of aestivation. In spite of urea synthesis being energy intensive and fish aestivating in hypoxia being confronted with inefficient energy metabolism, the author hypothesized that up-regulation of mRNA expression of OUC enzymes, specifically CPS III and glutamine synthetase (GS), would occur in fish aestivating in both normoxic and hypoxic conditions. This is because urea might have an important role in inducing aestivation in African lungfish (Ip et al., 2004c, d), and therefore increased urea synthesis could be intrinsic to the aestivation process independent of the conditions (normoxia/hypoxia/mud) under which aestivation occurred. In addition, the author aimed to test the hypothesis that, besides certain

OUC enzymes, the gene expression of some other enzymes involved in amino acid and carbohydrate metabolism in the liver would be differentially affected by aestivation in normoxia and aestivation in hypoxia. Finally, it was hoped that information obtained from this study would reveal novel gene clusters associated solely with aestivation (i.e., in normoxia), and gene clusters that would be induced through the combined effects of aestivation and hypoxia.

7.2. Materials and methods

7.2.1. Fish

P. annectens (80-120 g body mass) were imported from Central Africa through a local fish farm in Singapore. Specimens were maintained in plastic aquaria filled with dechlorinated water, containing 2.3 mmol l^{-1} Na^+, 0.54 mmol l^{-1} K^+, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^- and 0.6 mmol l^{-1} HCO_3^-, at pH 7.0 and at 25°C in the laboratory, and water was changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 month. During the adaptation period, fish were fed frozen fish meat.

7.2.2. Experimental conditions

Protopterus annectens were induced to aestivate in the presence of small volumes of water inside air-tight plastic containers continuously flushed with air (normoxia) or a calibrated gas mixture (2% O_2 in N_2; hypoxia) as described by Loong et al. (2008b; Chapter 3). Under standard laboratory conditions, the experimental fish would secrete mucus during the first few days, and the mucus would slowly dry up between day 6 and day 7 to form a mucus cocoon. In this study, the author focused on day 6, i.e. the end of the induction phase of aestivation. Fish were killed on day 6 and the liver excised and kept at -80^{o}C until further analysis. Control fish were kept in freshwater for 6 days.

7.2.3. Construction of SSH libraries

Total RNA was extracted from the liver, using chaotropic extraction protocol described by Whitehead and Crawford (2005). Frozen liver tissues were homogenized using an electric homogenizer (Pro Scientific Inc., Oxford, CT, USA) in 400 µl chaotropic buffer (4.5 M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50 mM EDTA (pH 8.0), 25 mM Tris-HCl (pH 7.5), 0.1 M β-mercaptoethanol, 0.2% antifoam A). All reagents were from

Sigma. Sodium acetate (2 M, pH 4.0) was added to the homogenate, following by 400 µl acidic phenol (pH 4.4), and 200 µl chloroform/isoamyl alcohol (23:1). The mixture was kept at 4°C for 10 min then centrifuged at 4°C at 10,000 x*g* for 20 min. Supernatant (400 µl) was removed and combined with 400 µl isopropanol, stored at -20°C overnight. Supernatant was centrifuged at 10,000 x*g* for 30 min at 4°C. The remaining RNA pellet was rinsed twice with 500 µl of 70% ethanol, then further purified using the Qiagen RNeasy Mini Kit (Qiagen Inc., CA, USA) following the manufacturer's protocols. RNA quality was checked electrophoretically by running 1 µg of RNA in 1% agarose gel. RNA quantification was done spectrophotometrically using Hellma traycell (Hellma GmbH & Co. KG, Müllheim, Germany). Two hundred µg of liver total RNA from each of the conditions was used for poly (A+) mRNA purification (Oligotek mRNA kit by Qiagen Inc., CA, USA)

SSH libraries were produced using PCR-Select^{TM} cDNA subtraction kit (Clontech, Takara Bio, Madison, USA), following the manufacturer's protocol. For each cDNA synthesis, 2 µg of poly(A)-selected mRNA was used per reaction. After second strand synthesis, the double stranded cDNA from both groups was digested with *Rsa I*. Part of the digested cDNA was ligated with Adapter 1 and part with Adaptor 2R, and the rest was saved for use as driver in preparation for hybridization. The forward library was made by hybridization adapter-ligated cDNA from 6 day of aestivation in normoxia or 6 day of aestivation in hypoxia (2% oxygen) in the presence of cDNA from the 0 day freshwater (control) fish as the driver. This forward reaction library was designed to produce clones that are up-regulated in the 6 day aestivated fish in normoxia or hypoxia relative to the control. The reverse library was made the same way, except that the adapter-ligated cDNA from the control fish serve as the tested and was hybridized in the presence of cDNA from the 6 day aestivated fish in normoxia or hypoxia as the driver. This reverse reaction library was designed to produce clones that are down-regulated in the aestivated fish (normoxia or hypoxia) relative to the control. In either case the driver cDNA was added in excess during

each hybridization to remove common cDNA by hybrid selection, leaving overexpressed and novel tester cDNAs to be recovered and cloned. PCR amplification of the differentially expressed cDNAs was performed with an Advantage cDNA polymerase mix (Clontech, Takara Bio, Madison, USA). Primary and secondary PCR amplification of these reciprocal subtractions of cDNA from control and aestivated (normoxia or hypoxia) groups produced 4 SSH libraries enriched in differentially expressed transcripts.

Differentially expressed cDNAs were cloned using pGEM Easy T/A cloning kit (Promega Corporation, Madison, WI, USA), transformed into *Escherichia coli* JM109 strain, and plated onto Luria-Bertani (LB) agar with ampicillin, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and isopropyl-beta-thio galactopyranoside (IPTG). Selected while colonies were grown overnight in LB with ampicillin. The plasmids were extracted using the resin-based plasmid miniprep kit (Axygen Biosciences, Union City, CA, USA). After quantification by spectrophotometry, plasmids were diluted to working concentrations. Approximately 80-100 ng of plasmid DNA was used in Big dye terminator reaction (Applied Biosystems, CA, USA) with 2 µM of T7 primers. Excess fluorescent nucleotides and salts were removed from the samples by ethanol precipitation. The dried samples were resuspended in Hi-Di Formamide for loading on the Prism^{TM} 3130XL sequencer (Applied Biosystems, CA, USA). A total of 500 clones for each forward and reverse library were selected for sequencing.

Sequence output was exported as text and edited manually to remove vector sequences. Trimmed sequences were uploaded to the BLAST server at the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (NLM), National Institutes of Health (NIH) website. BLAST searches were performed using the tBLASTx algorithim (Altschul et al., 1990) and default search conditions. Proteins were considered significant when the E-value was < 1e-04. Classification of the annotated sequences was searched by Mouse Genome Informatics and Gene Ontology.

7.3. Results

7.3.1. Six days aestivation in normoxia

7.3.1.1 Subtractive libraries

Two subtracted libraries, forward (Table 7.1) and reverse (Table 7.2), were constructed to reveal the up-regulation and down-regulation of genes, respectively, in the liver of *P. annectens* in response to 6 days of aestivation in normoxia. A total of 641 genes were identified from the two subtracted libraries: forward 316 and reverse 325. The rest of the sequences (359/1,000) had no matches in available databases. These sequences could be unknown *P. annectens* genes and they will be characterized in future studies. Very few genes were identified in either the forward and reverse libraries from the same tissue and condition, indicating that subtraction by hybridization was quite effective.

7.3.1.2 Forward library (up-regulation)

There were up-regulation of *cps*, *ass*, and *gs* genes in the liver of *P. annectens* aestivated for 6 days in normoxia (Table 7.1). Some genes coding for proteins involved in fatty acid metabolism and complement activation were up-regulated. Other up-regulated genes include tissue factor pathway inhibitor, those coding for protein involved in iron and copper metabolism, specific types of haemoglobins (epsilon and gamma), and those coding for ribosomal proteins and translation factors (Table 7.1).

7.3.1.3 Reverse library (down-regulation)

There were down-regulation of genes related to carbohydrate metabolism (Table 7.2). Some genes involved in complement activation and blood coagulation were also down-regulated. Other down-regulated genes include those involved those in iron and copper metabolism, fatty acid binding, and those related to ribosomes and translation (Table 7.2).

7.3.2 Six days of aestivation in hypoxia

7.3.2.1 Subtractive libraries

Similarly, a forward (Table 7.3) and a reverse (Table 7.4) libraries were constructed to reveal the up-regulation and down-regulation of genes, respectively, in the liver of *P. annectens* in response to 6 days of aestivation in hypoxia. A total of 733 genes were identified from the 2 subtracted libraries: forward 362 and reverse 371. The rest of the sequences (267/1,000) had no matches in available databases.

7.3.2.2 Forward library

7.3.2.2.1 Similarities to normoxia

Similar to normoxia (Table 7.1), there were up-regulations of *cps*, *ass*, and *gs* in the liver of *P. annectens* in hypoxia (Table 7.3). Stearoyl-Co A synthetase gene, mannan-binding lectin associated serine protease 1 (*masp1*) and *cfb* were up-regulated in both conditions. Other genes up-regulated in both normoxia (Table 7.1) and hypoxia (Table 7.3) include those involved in complement activation, tissue factor pathway inhibitor, ferritin heavy chain, transferrin, ceruloplasmin, haemoglobin gamma and haemoglobin epsilon, and those coding for ribosomal protein and 16s rRNA (Table 7.3).

7.3.2.2.2 Differences from normoxia

There were marked differences in the up-regulation of genes related to carbohydrate metabolism between normoxia (Table 7.1) and hypoxia (Table 7.3). While normoxia did not induce up-regulation of genes in carbohydrate metabolism, hypoxia lead to up-regulation of genes coding for fructose 1,6-bisphosphatase, fructose-2,6-bisphosphatase, aldolase, phosphoenolpyruvate caroboxykinase, and lactate dehydrogenase. Different from normoxia (Table 7.1), genes related to translation factors (eif1b, eif6, eef1a1, eef1g, and etf1) were down-regulated in hypoxia (Table 7.3).

7.3.2.3 Reverse library

7.3.2.3.1 Similarities to normoxia

Genes down-regulated in both normoxia (Table 7.2) and hypoxia (Table 7.4) include aldolase B, complement component 3, fibrinogen beta chain, and *serpinc1*. Transferrin was the only gene related to iron metabolism that was down-regulated in both conditions. Similar to normoxia (Table 7.2), genes coding for ribosomal protein and eif3e were down-regulated in hypoxia (Table 7.4).

7.3.2.3 Reverse library

7.3.2.3.2 Differences from normoxia

Unlike normoxia where there were down-regulation of enolase, glyceraldehyde-3-phsophate dehydrogenase, fructose-1,6-bisphosphatase and lactate dehydrogenase (Table 7.2), there was only down-regulation of malate dehydrogenase in hypoxia (Table 7.4). Ceruloplasmin and haemoglobin epsilon were down-regulated in hypoxia (Table 7.4). Unlike normoxia where there were down-regulation of 21 ribosomal genes and 3 translation elongation factors (Table 7.2), in hypoxia there were down-regulation of 4 genes coding for ribosomal protein and 1 translation initiation factor (Table 7.4).

Table 7.1. Known transcripts found in the forward SSH library of liver of *P. annectens* aestivated for 6 days in normoxia.

Group	Gene	*Species*	Biological processes	E-value	No of clones
Nitrogen metabolism					
	Argininosuccinate synthetase 1 (Ass1)	*Danio rerio*	Amino acid biosynthetic process. Arginine biosynthetic process	1.00E-33	18
	Carbamoyl-phosphate Synthetase (Cps)	*Squalus acanthias*	Glutamine metabolic process	2.00E-112	2
	Glutamine Synthetase (Gs)	*Xenopus laevis*	Glutamine biosynthetic process. Nitrogen compound metabolic process	7.00E-77	10
Amino acid, polyamine and nucleotide metabolism					
	Acetylserotonin O-methyltransferase (Asmt)	*Taeniopygia guttata*	Melatonin biosynthetic process	4.00E-09	1
	Glutathione S-transferase M	*Danio rerio*	Metabolic process	5.00E-46	1
	Homogentisate 1, 2-dioxygenase (Hgd)	*Taeniopygia guttata*	Amino acid metabolic process. L-phenylalanine catabolic process	5.00E-104	3
	Inter-alpha trypsin inhibitor heavy chain 3	*Taeniopygia guttata*	Hyaluronan metabolic process	5.00E-25	2
	Retinol binding protein 1 (Rbp)	*Cyprinus carpio*	Retinoic acid metabolic process.	7.00E-40	2
Lipoprotein, fatty acid and cholesterol metabolism and transport					
	Apolipoprotein A-IV (Apoa4)	*Xenopus tropicalis*	Lipid transport. Innate immune response in mucosa	3.00E-16	3
	Apolipoprotein B (Apob)	*Homo sapiens*	Artery morphogenesis. Cholesterol homeostasis	4.00E-13	5
	Fatty acid binding protein 1, liver (Fabp1)	*Danio rerio*	Transport	9.00E-21	2
	Stearoyl-coA desaturase (Scd1)	*Salmo salar*	Brown fat cell differentiation. Fatty acid biosynthetic process	2.00E-32	8
Complement					
	Complement factor B (Cfb)	*Homo sapiens*	Complement-mediated immunity. Cell proliferation	7.00E-08	7
	Complement component C4	*Oncorhynchus mykiss*	Complement-mediated immunity	4.00E-29	1
	Complement component C7	*Danio rerio*	Complement-mediated immunity	4.00E-14	2
	Complement component factor I (Cfi)	*Xenopus laevis*	Complement activation. Immune response	5.00E-27	1
	Mannan-binding lectin-associated serine protease 1 (Masp1)	*Cyprinus carpio*	Complement activation. Immune response	4.00E-29	7
Blood coagulation					
	Tissue factor pathway inhibitor 2 (Tfpi2)	*Danio rerio*	Blood coagulation	3.00E-29	9
Iron, copper metabolism and transport					
	Ceruloplasmin (Cp)	*Taeniopygia guttata*	Copper ion transport	1.00E-48	11
	Ferritin heavy chain 1 (Fth1)	*Bufo gargarizans*	Iron ion transport	2.00E-84	23
	Haemoglobin gamma G	*Homo sapiens*	Oxygen transport	6.00E-22	2
	Haemoglobin, epsilon 1 (Hbe1)	*Taeniopygia guttata*	Oxygen transport	1.00E-20	2

Table 7.1 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Iron, copper metabolism and transport					
	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9 (Ndufb9)	*Danio rerio*	Electron transport chain	4.00E-34	2
	Transferrin (Trf)	*Xenopus laevis*	Iron transport	4.00E-61	7
Response to stimulus					
	Heat shock protein 20	*Ostertagia ostertagi*	Stress response. Response to heat	5.00E-30	14
Protein synthesis, transport and folding					
	Alpha 1 microglobulin/bikunin (Ambp)	*Xenopus tropicalis*	Protein maturation. Protein-chromophore linkage	4.00E-21	7
	Mitochondrially encoded 16S rRNA (mt-Rnr2)	*Protopterus annectens*	Ribosomal large subunit assembly	1.00E-175	1
	Eukaryotic translation elongation factor 2 (Eef2)	*Ornithorhynchus anatinus*	GTP binding. GTPase activity	5.00E-85	3
	Eukaryotic translation initiation factor 3 subunit C (Eif3c)	*salmo salar*	Translational initiation	1.00E-105	7
	Ribosomal protein SA (Rpsa)	*Homo sapiens*	Translation	5.00E-97	5
	Ribosomal protein S2 (Rps2)	*Taeniopygia guttata*	Translation	2.00E-97	1
	Ribosomal protein S13 (Rps13)	*Rattus norvegicus*	Translation	3.00E-105	1
	Ribosomal protein L3 (Rpl3)	*Xenopus laevis*	Translation	5.00E-117	1
	Ribosomal protein L7a (Rpl7a)	*Protopterus dolloi*	Ribosome biogenesis	2.00E-76	5
	Ribosomal protein L11 (Rpl11)	*Protopterus dolloi*	Translation	1.00E-105	1
	Ribosomal protein L12 (Rpl12)	*Xenous laevis*	Translation	6.00E-102	1
	Ribosomal protein L13a (Rpl13a)	*Xenopus laevis*	Translation	3.00E-81	3
	Ribosomal protein L19 (Rpl19)	*Protopterus dolloi*	Translation	1.00E-91	1
	Ribosomal protein L35a (Rpl35a)	*Mus musculus,*	Translation	5.00E-85	1
	Ribosomal protein, large P1 (Rplp1)	*Salmo salar*	Translational elongation	1.00E-30	2
	Ubiquitin-conjugating enzyme E2M (Ube2m)	*Xenopus laevis*	Modification-dependent protein catabolic process, post-translational	9.00E-144	6
Structure					
	Crystallin, alpha A (Cryaa)	*Caiman crocodilus*	Actin filament organization. Camera-type eye development	3.00E-08	13
	Stomatin (Stom)	*Danio rerio*	Cell structure and motility	8.00E-50	2
Signaling					
	Guanine nucleotide binding protein (G protein) beta polypeptide (Gnb2l1)	*Protopterus dolloi*	Intracellular signalling cascade. Protein localization	5.00E-103	23
	Probable signal peptidase complex subunit 2 (Spcs2)		Signal peptide processing	1.00E-08	1

Table 7.1 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Signaling					
	Tachykinin receptor protein 1 (Tacr1)	*Neoceratodus forsteri*	G-protein coupled receptor protein signalling pathway; response to pain	8.00E-148	7
Transcription					
	Basic leucine zipper and W2 domains 1 (Bzw1)	*Rattus norvegicus*	Regulation of transcription	3.00E-103	6
	Heterogeneous nuclear ribonucleoprotein D-like (Hnrpdl)	*Taeniopygia guttata*	Regulation of transcription	1.00E-17	1
	Polyglutamine binding protein 1 (Pqbp1)	*Homo sapiens*	Regulation of transcription. DNA-dependent transcription	4.00E-07	25
	Reverse transcriptase/ribonuclease H/putative methyltransferase	*Danio rerio*	Reverse transcription. Proteolysis	2.00E-10	5
	X box binding protein 1 (Xbp1)	*Xenopus laevis*	DNA-dependent transcription	2.00E-14	9
mRNA processing					
	Y box binding protein 1 (Ybx1)	*Rattus norvegicus*	mRNA processing. In utero embryonic development	2.00E-37	3
Cell cycle					
	Annexin A1 (Anxa1)	*Xenopus laevis*	Cell cycle. Arachidonic acid secretion	4.00E-22	2
Chromosome and DNA					
	Histone family, member Z (H2afz)	*Taeniopygia guttata*	Nucleosome assembly. Multicellular organismal development	5.00E-122	3
	Topoisomerase (DNA) I (Top1)	*Taeniopygia guttata*	DNA replication, DNA topological change	3.00E-13	2
Others					
	Brain protein 44 (Brp44)	*Xenopus tropicalis*	Unclassified	3.00E-42	1
	Endonuclease domain containing 1 (Endod1)	*Danio rerio*	Unclassified	1.00E-06	5
	Repeat sequence LfR1 LINE	*Lepidosiren paradoxa*	Unclassified	2.00E-17	8
	Tetratricopeptide repeat domain 36 (Ttc36)	*Taeniopygia guttata*	Unclassified	1.00E-38	3
	Ribosomal protein L26 (Rpl26)	*Danio rerio*	Others	7.00E-24	6

Table 7.2. Known transcripts found in the reverse SSH library of liver of *P. annectens* aestivated for 6 days in normoxia.

Group	Gene	*Species*	Biological processes	E-value	No of clones
Carbohydrate metabolism					
	Enolase 1 (alpha) (Eno1)	*Xenopus tropicalis*	Glycolysis	3.00E-118	2
	Fructose-1,6-bisphosphatase 1 (Fbp1)	*Danio rerio*	Gluconeogenesis	1.00E-23	1
	Fructose-bisphosphate, aldolase B (Aldob)	*Protopterus annectens*	Glycolysis	1.00E-70	2
	Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	*Protopterus annectens*	Glycolysis	9.00E-69	3
	Lactate dehydrogenase A (Ldha)	*Xenopus laevis*	Carbohydrate metabolic process	6.00E-128	2
	Transaldolase 1 (Taldo1)	*Xenopus laevis*	Carbohydrate metabolic process	2.00E-48	1
Amino acid, polyamine and nucleotide metabolism					
	Aminolevulinic acid synthase 1 (Alas1)	*Protopterus dolloi*	Heme biosynthetic process	8.00E-76	1
	Betaine-homocysteine methyltransferase (Bhmt)	*Xenopus tropicalis*	Methionine biosynthetic process	1.00E-112	3
	Folate receptor 1 (Folr1)	*Taeniopygia guttata*	Folic acid metabolic process, posttranslational protein targeting to membrane	5.00E-15	1
	Glutathione S-transferase pi 1 (Gstp1)		Glutathione metabolic process, metabolic process.	5.00E-31	3
	Glyoxylate reductase/hydroxypyruvate reductase (Grhpr)	*Xenopus laevis*	Metabolic process, oxidation reduction	1.00E-34	1
	Inter-alpha trypsin inhibitor heavy chain 2 (Itih2)	*Taeniopygia guttata*	Hyaluronan metabolic process	2.00E-69	2
	Inter-alpha trypsin inhibitor heavy chain 3 (Itih3)	*Danio rerio*	Hyaluronan metabolic process	8.00E-30	2
	Nucleoside diphosphate kinase	*Taeniopygia guttata*	Pyrimidine metabolism	1.00E-17	1
	Ornithine decarboxylase antizyme (Odcaz)	*Taeniopygia guttata*	Polyamine metabolic process	3.00E-145	2
Complement					
	Complement component 1, q subcomponent, beta polypeptide (C1qb)	*Protopterus aethiopicus*	Complement activation. Immune response	2.00E-147	2
	Complement component C3	*Taeniopygia guttata*	Complement activation. G-protein coupled receptor protein signalling pathway	3.00E-20	1
	Complement component 8, alpha polypeptide (C8a)	*Xenopus tropicalis*	Complement activation	8.00E-39	3
Blood coagulation					
	Apolipoprotein H (Apoh)	*Salmo salar*	Regulation of blood coagulation	7.00E-45	2
Blood coagulation					
	Coagulation factor 2 (F2)	*Taeniopygia guttata*	Acute-phase response. Blood coagulation	3.00E-34	11
	Fibrinogen beta chain (Fgb)	*Danio rerio*	Blood coagulation, platelet activation	2.00E-50	3
	Fibrinogen gamma polypeptide (Fgg)	*Xenopus tropicalis*	Blood coagulation, platelet activation	1.00E-73	1
	Plasminogen (Plg)	*Danio rerio*	Apoptosis, Blood coagulation	3.00E-22	1

Table 7.2 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Blood coagulation					
	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1 (Serpinc1)	*Xenopus laevis*	Blood coagulation	6.00E-63	4
Iron, copper and vitamin metabolism and transport					
	Ferritin heavy chain (Fth1)	*Taeniopygia guttata*	Iron ion transport	1.00E-23	3
	Ferritin light chain (Ftl1)	*Salmo salar*	Iron ion transport	1.00E-11	2
	Transferrin (Trf)	*Oncorhynchus mykiss*	Cellular iron ion homeostasis	1.00E-54	2
	Retinol binding protein 4	*Ornithorynchus anatinus*	Vitamin/cofactor transport	1.00E-37	1
Lipoprotein metabolism and transport					
	Alpha 1 microglobulin/bikunin	*Xenopus tropicalis*	Proteolysis; Fatty acid biosynthesis; Lipid metabolism; Intracellular signaling cascade; Transport	1.00E-26	1
	Apolipoprotein A-IV precursor (Apolipoprotein A-IV) (Apoa4)	*Salmo salar*	Lipid transport. Innate immune response in mucosa	7.00E-42	2
	Fatty acid binding protein 1, liver (Fabp1)	*Xenopus laevis*	Transport	2.00E-64	11
Transport					
	ADP/ATP translocase (AAT)	*Taeniopygia guttata*	Nucleoside, nucleotide and nucleic acid transport; Transport	5.00E-20	2
	Albumin (Alb)	*Danio rerio*	Transport	2.00E-07	1
	Amyloid beta (A4) precursor-like protein-binding, family A, member 3 (Apba3)	*Rana catesbeiana*	Protein transport. in utero embryonic development	1.00E-88	11
	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9) (Atp5g1)	*Taeniopygia guttata*	Ion transport. ATP synthesis coupled protein transport	6.00E-19	1
	Solute carrier family 25 (Adenine nucleotide translocase) member 5	*Rana amurensis*	Transport	6.00E-38	1
Transport					
	Mannose-specific lectin 1 (Lman1)	*Taeniopygia guttata*	Protein transport. ER to Golgi vesicle-mediated transport	2.00E-61	2
Transport					
	Solute carrier organic anion transporter family, member 1A2	*Taeniopygia guttata*	Glycoprotein. Organic anion transport	6.00E-83	3
Response to stimulus					
	Cytoglobin (Cygb)	*Danio rerio*	Response to oxidative stress. Oxygen transport	8.00E-09	1
	Serine (or cysteine) proteinase inhibitor, clade A, member 1d (Serpina1d)	*Xenopus laevis*	Response to cytokine stimulus. Response to peptide hormone	3.00E-18	3

Table 7.2 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Response to stimulus					
	Serine (or cysteine) peptidase inhibitor, clade F , member 2 (Serpinf2)	*Danio rerio*	Acute-phase response	3.00E-19	2
	Serpin (or cysteine) peptidase inhibitor, clade A, member 1 (Serpina1)	*Xenopus laevis*	Response to cytokine stimulus, response to peptide hormone stimulus...	5.00E-58	4
Response to stimulus					
	Heat shock protein 20	*Ostertagia ostertagi*	Stress response. Response to heat	2.00E-07	4
	Lipocalin 1 (Lcn1)	*Bufo marinus*	Response to stimulus, Transport, Sensory perception of tast. Proteolysis	2.00E-09	4
Cell adhesion					
	Lectin, galactose binding, soluble 1 (Lgals1)	*Danio rerio*	Heterophilic cell adhesion. Myoblast differentiation	1.00E-10	1
	Nidogen 2 (Nid2)	*Danio rerio*	Cell adhesion. Cell-matrix adhesion	1.00E-42	1
	Lectin, galactose binding, soluble 1 (Lgals1)	*Danio rerio*	Myoblast differentiation. Heterophilic cell adhesion	2.00E-56	5
Protein synthesis, transport, folding and modification					
	Heme-binding protein 2 (Hebp2)	*Danio rerio*	Protein biosynthesis	5.00E-25	1
	Eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	*Ornithorynchus anatinus*	Translational regulation	3.00E-170	1
	Eukaryotic translation elongation factor 1 gamma (Eef1g)	*Salmo salar*	Translational elongation	2.00E-47	1
	Eukaryotic translation elongation factor 2 (Eef2)	*Taeniopygia guttata*	GTP binding. GTPase activity	1.00E-45	1
	Ribosomal protein SA (Rpsa)	*Protopterus dolloi*	Translation	4.00E-41	1
	Ribosomal protein S5 (Rps5)	*Taeniopygia guttata*	Translation	2.00E-71	3
	Ribosomal protein S6 (Rps6)	*Xenopus laevis*	Activation-induced cell death of T cells. Erythrocyte development	1.00E-32	1
	Ribosomal protein S10 (Rps10)	*Ornithorynchus anatinus*	Translation	5.00E-126	2
	Ribosomal protein S12 (Rps12)	*Danio rerio*	Translation	6.00E-20	4
	Ribosomal protein S16 (Rps16)	*Xenopus laevis*	Translation	7.00E-41	2
	Ribosomal protein S17 (Rps17)	*Taeniopygia guttata*	Translation	2.00E-79	1
	Ribosomal protein S18 (Rps18)	*Protopterus dolloi*	Translation	3.00E-14	2
	Ribosomal protein S26 (Rps26)	*Rattus norvegicus*	Translation	2.00E-21	1
	Ribosomal protein L7 (Rpl7)	*Cyprinus carpio*	Translation	7.00E-06	1
	Ribosomal protein L9 (Rpl9)	*Protopterus dolloi*	Translation	4.00E-167	4
	Ribosomal protein L13 (Rpl13)	*Taeniopygia guttata*	Translation	3.00E-27	1
	Ribosomal protein L18 (Rpl18)	*Salmo salar*	Translation	5.00E-47	2
	Ribosomal protein L19 (Rpl19)	*Danio rerio*	Translation	7.00E-47	1
	Ribosomal protein L28 (Rpl28)	*Mus musculus*	Translation	7.00E-73	3

Table 7.2 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Protein synthesis, transport, folding and modification					
	Ribosomal protein L29	*Ictalurus punctatus*	Cell proliferation. Cell-substrate adhesion	2.00E-32	1
	Ribosomal protein L32 (Rpl32)	*Protopterus dolloi*	Translation	0	2
	Ribosomal protein L35 (Rpl35)	*Xenopus tropicalis*	Translation	1.00E-15	2
	Ribosomal protein L35a (Rpl35a)	*Taeniopygia guttata*	Translation	3.00E-67	1
	Ribosomal protein L37 (Rpl37)	*Mus musculus*	Translation	7.00E-79	1
	Ribosomal protein L41 (Rpl41)	*Taeniopygia guttata*	Translation	2.00E-64	1
	Ribosomal protein Large P0 (Rplp0)	*Xenopus laevis*	Translational elongation	4.00E-60	1
	Ribosomal protein, large, P1 (Rplp1)	*Protopterus dolloi*	Translational elongation	2.00E-130	2
	Ribosomal protein large P2 (Rplp2)	*Protopterus dolloi*	Translational elongation	4.00E-89	3
	Threonyl-tRNA synthetase-like 2 (Tarsl2)	*Protopterus dolloi*	Threonyl-tRNA aminoacylation, Translation	5.00E-94	1
	Ubiquitin C (Ubc)	*Taeniopygia guttata*	Protein modification process	4.00E-84	1
	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6 (core 3 synthase) (B3dnt6)	*Ornithorynchus anatinus*	Protein amino acid glycosylation	1.00E-72	1
Signaling					
	Ephrin receptor A1 (Epha1)	*Taeniopygia guttata*	Protein amino acid phosphorylation, transmembrane receptor protein tyrosine kinase signaling pathway	1.00E-29	1
Signaling					
	KH domain containing, RNA binding, signal transduction associated 1	*Taeniopygia guttata*	Cell cycle, cell surface receptor linked signal transduction	3.00E-60	2
Structural					
	Actin, beta	*Xenopus laevis*	Exocytosis; endocytosis; Transport; Cytokinesis; Cell structure	0	1
	Crystallin, alpha A (Cryaa)	*Caiman crocodilus*	Actin filament organization. Camera-type eye development	9.00E-08	5
Cell growth, cycle and proliferation					
	Selenoprotein P, plasma 1 (Sepp1)	*Xenopus tropicalis*	Growth. Brain development	1.00E-21	1
	Annexin A1 (Anxa1)	*Rana catesbeiana*	Arachidonic acid secretion, cell cycle	1.00E-25	3
	RNA binding motif protein 5 (Rbm5)	*Taeniopygia guttata*	Cell cycle, negative regulation of cell cycle	2.00E-83	4
	Parkinson disease 7 (Park7)	*Taeniopygia guttata*	Adult locomotory behavior, cell proliferation.	3.00E-84	1
Transcription					
	Polyglutamine binding protein 1 (Pqbp1)	*Homo sapiens*	Regulation of transcription. DNA-dependent transcription	1.00E-08	26

Table 7.2 Continued

Group	Gene	*Species*	Biological processes	E-value	No of clones
Oxidation reduction					
	NADH dehydrogenase (ubiquione) Fe-S protein 8 (Ndufs8)	*Xenopus tropicalis*	Oxidation reduction. Electron transport chain	3.00E-38	1
	Phosphogluconate dehydrogenase (Pgd)	*Ornithorynchus anatinus*	Oxidation reduction	5.00E-66	1
	Sorbitol dehydrogenase (Sord)	*Xenopus tropicalis*	Oxidation reduction. Sorbitol metabolic process	2.00E-51	3
	Cytochrome P450, family 2, subfamily J, polypeptide 2 (Cyp2f2)	*Mus musculus*	Oxidative reduction. Napthalene metabolic process	7.00E-27	1
Others					
	Apolipoprotein AI (Apoa1bp)	*Salmo salar*	Unclassified	9.00E-49	1
	Brain protein 44 (Brp44)		Unclassified	1.00E-08	1
	Complement component 4A	*Xenopus tropicalis*		6.00E-48	1
	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21 (Ddx21)	*Danio rerio*	Unclassified	1.00E-61	3
	Fetuin beta (Fetub)	*Xenopus tropicalis*	Unclassified	4.00E-20	92
	Lectin precursor	*Salmo salar*	Unclassified	8.00E-13	1
	Novel pentaxin family domain containing protein	*Danio rerio*	Unclassified	5.00E-37	1
	Nucleic acid binding protein RY-1 variant 4-like & ATP synthase, H+ transporting, mitochondrial F0 complex subunit C2	*Taeniopygia guttata*	Unclassified	6.00E-19	1
	Repeat sequence LfR1 LINE	*Lepidosiren paradoxa*	Unclassified	2.00E-23	1

Table 7.3. Known transcripts found in the forward SSH library of liver of *P. annectens* aestivated for 6 days in hypoxia.

Group	Gene (Symbol)	*Species*	Biological processes	E-value	No of clones
Nitrogen Metabolism					
	Argininosuccinate synthetase 1 (Ass1)	*Danio rerio*	Amino acid biosynthetic process. Arginine biosynthetic process	5.00E-34	14
	Carbamoyl phosphate synthetase (Cps)	*Danio rerio*	Glutamine metabolic process	1.00E-124	6
	Glutamate Synthetase (Gs)	*Xenopus laevis*	Glutamine biosynthetic process. Nitrogen compound metabolic process	7.00E-108	9
Carbohydrate Metabolism					
	Fructose-1,6-bisphosphatase 1 (Fbp1)	*Xenopus tropicalis*	Carbohydrate metabolic process. Gluconeogenesis	2.00E-43	3
	Fructose-2,6-biphosphatase 4 (Pfkfb4)	*Homo sapiens*	Fructose metabolic process	2.00E-76	1
	Fructose-bisphosphate aldolase A (Aldoa)	*Protopterus annectens*	Glycolysis. Metabolic process	4.00E-40	2
	Fructose-bisphosphate aldolase B (Aldob)	*Protopterus annectens*	Glycolysis. Metabolic process	2.00E-96	1
	Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	*Protopterus annectens*	Glucose metabolic process. Glycolysis	0	4
	Lactate dehydrogenase A (Ldha)	*Danio rerio*	Carbohydrate metabolic process	6.00E-101	1
	Phosphoenolpyruvate carboxykinase (PEPCK-C)	*Xenopus tropicalis*	Gluconeogenesis. Glycerol biosynthetic process from pyruvate	5.00E-81	2
	Triose phosphate isomerase	*Protopterus annectens*	Glycolysis.	0	1
Amino Acid, polyamine and nucleotide Metabolism					
	Dihydrodiol dehydrogenase (dimeric) (Dhdh)	*Salmo salar*	Metabolic process. Oxidation reduction	1.00E-31	3
	Glutathione S-transferase alpha 3 (Gsta3)	*Taeniopygia guttata*	Metabolic process	2.00E-38	2
	Hemopexin (Hpx)	*Rattus norvegicus*	Heme metabolic process. Haemoglobin metabolic process	2.00E-08	1
	Homogentisate 1,2-dioxygenase (Hgd)	*Danio rerio*	Amino acid metabolic process. L-phenylalanine catabolic process	3.00E-85	2
	Matrix metalloproteinase 7 (Mmp7)	*Taeniopygia guttata*	Collagen catabolic process. Metabolic process	6.00E-31	2
	Retinol binding protein 1 cellular(rbp)	*Cyprinus carpio,*	Retinoic acid metabolic process.	2.00E-45	1
	Thiamin pyrophosphokinase 1	*Homo sapiens*	Thiamine diphosphate biosynthetic process. Thiamin metabolic process	1.00E-34	1
Fatty acid biosynthesis					
	Prostaglandin D Synthase (Ptgds)	*Xenopus laeivs*	Fatty acid biosynthetic process. Lipid biosynthetic process	7.00E-11	4

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Fatty acid biosynthesis					
	Stearoyl-CoA desaturase (Scd1)	*Salmo salar*	Brown fat cell differentiation. Fatty acid biosynthetic process	2.00E-37	3
Catabolism					
	Myeloperoxidase (Mpo)	*Homo sapiens*	Hydrogen peroxide catabolic process	9.00E-13	1
	Ornithine decarboxylase 1 (Odc1)	*Taeniopygia guttata*	Kidney development. Polyamine biosynthetic process	2.00E-34	1
Catabolism					
	Peroxiredoxin 3 (Prdx3)	*Mus musculus,*	Cell redox homeostasis. Hydrogen peroxide catabolic process	7.00E-93	1
	Thyroid peroxidase (Tpo)	*Homo sapiens*	Hormone biosynthetic process. Hydrogen peroxide catabolic process	3.00E-17	1
	Valosin containing protein (Vcp)	*Danio rerio*	Activation of caspase activity. Modification-dependent protein catabolism	1.00E-35	5
Lipoprotein, fatty acid and cholesterol homeostasis and transport					
	Apolipoprotein B (Apob)	*Gallus gallus*	Artery morphogenesis. Cholesterol homeostasis	1.00E-16	2
	Lipase, hepatic (Lipc)	*Xenopus tropicalis*	Cholesterol homeostasis. Cholesterol metabolic process	4.00E-28	2
	Apolipoprotein A-IV (Apoa4)	*Homo sapiens*	Lipid transport. Innate immune response in mucosa	8.00E-51	8
Complement					
	Complement factor B (Cfb)	*Rattus norvegicus*	Complement-mediated immunity. Cell proliferation	2.00E-20	3
	Complement component C3	*Ornithorhynchus anatinus*	Complement activation. G-protein coupled receptor protein signalling pathway	1.00E-54	3
	Complement component C4	*Cyprinus carpio*	Complement-mediated immunity	5.00E-07	1
	Complement factor H-related protein	*Ornithorhynchus anatinus*	Complement activation	4.00E-08	2
	Complement component 1, r subcomponent (C1r)	*Taeniopygia guttata*	Complement mediated immunity. Proteolysis	1.00E-52	1
Complement					
	Mannan-binding lectin associated serine protease 1(Masp1)	*Taeniopygia guttata*	Complement activation. Immune response	3.00E-19	1
Blood coagulation					
	Apoliprotein H (Apoh)	*Salmo salar*	Regulation of blood coagulation	3.00E-40	1

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Blood coagulation					
	Coagulation factor IX (F9)	*Orrnithorhynchus anatinus*	Blood coagulation. Proteolysis	1.00E-08	1
	Coagulation factor XIII, Beta subunit (F13b)	*Ornithorhynchus anatinus*	Blood coagulation	1.00E-14	1
	Fibrinogen alpha chain (Fga)		Blood coagulation	3.00E-14	1
	Serine (or cysteine) peptidase inhibitor, clade D, member 1 (Serpind1) (heparin cofactor II)	*Xenopus laevis*	Blood coagulation	5.00E-53	2
	Plasminogen (Plg)	*Homo sapiens*	Apoptosis. Blood coagulation	1.00E-38	2
	Protein C (inactivator of coagulation factors Va and VIIIa)	*Xenopus laevis*	Blood coagulation. Negative regulation of apoptosis	2.00E-58	1
	Tissue factor pathway inhibitor (Tfpi)	*Danio rerio*	Blood coagulation	4.00E-40	4
Iron, copper metabolism and transport					
	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (Atp5g3)	*Xenopus tropicalis*	Ion transport. ATP synthesis coupled protein transport	6.00E-26	1
	Ceruloplasmin (Cp)	*Danio rerio*	Copper ion transport	1.00E-54	5
	Ferritin heavy chain 1 (Fth1)	*Bufo gargarizans*	Iron ion transport	1.00E-84	14
	Ferritin light chain (Ftl1)	*Xenopus laevis*	Iron ion transport	3.00E-50	11
	Haemoglobin, epsilon 1 (Hbe1)	*Xenopus tropicalis*	Oxygen transport	7.00E-14	2
	Haemoglobin, gamma G	*Homo sapiens*	Oxygen transport	7.00E-15	1
	Mitochondrial carrier, adenine nucleotide translocator	*Taeniopygia guttata*	Transport	1.00E-29	7
	Transferrin (Trf)	*Xenopus laevis*	Iron transport	9.00E-31	14
	Tumor necrosis factor, alpha-induced protein 9 (STEAP4)	*Taeniopygia guttata*	Iron ion transport	8.00E-34	4
Iron, copper metabolism and transport					
	Coatomer protein complex, subunit beta 1 (Copb1)	*Taeniopygia guttata*	Intracellular protein transport.	2.00E-71	2
	RAN, member RAS oncogene family	*Taeniopygia guttata*	Intracellular protein transport. Nucleocytoplasmic transport	4.00E-41	1
	Solute carrier family 20 (phosphate transporter), member 1	*Xenopus tropicalis*	Phosphate transport	2.00E-21	1
	Transthyretin (Ttr)	*Xenopus laevis*	Thyroid hormone generation. Transport	1.00E-10	4

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Response to Stimulus					
	Cold inducible RNA binding protein-like	*Taeniopygia guttata*	Response to stress	2.00E-31	6
	Heat shock protein 20	*Ostertagia ostertagi*	Unclassified	4.00E-05	10
	Serine (or cysteine) peptidase inhibitor, clade A, member 3M (Serpina3m)	*Mus musculus*	Response to cytokine stimulus. Response to peptide hormone stimulus	4.00E-20	1
	Serine (or cysteine) proteinase inhibitor, clade A, member 1d (Serpina1d)	*Xenopus laevis*	Blood coagulation	2.00E-54	3
Cytolysis					
	Perforin-1 precursor (P1)	*Danio rerio*	Cytolysis	9.00E-24	1
Defense					
	Liver-expressed antimicrobial peptide 2 (Leap2)	*Danio rerio*	Defense response to bacterium	5.00E-14	2
Detoxification					
	Superoxide dismutase 1 (Sod1)	*Mus musculus*	Activation of MAPK actvitiy. Aging	3.00E-35	6
Apoptosis					
	Translationally-controlled tumor protein 1 (Tpt1)	*Mus musculus,*	Anti-apoptosis	1.00E-13	3
Binding					
	APOBEC-1 complementation factor (A1cf)	*Homo sapiens*	Cytidine to uridine editing. mRNA processing	6.00E-89	2
	Glycyl-tran synthetase (Gars)	*Homo sapiens*	Glycyl-tRNA aminoacylation. Regulated secretory pathway	2.00E-65	1
Cell adhesion					
	Aggrecan (Acan)	*Danio rerio*	Cartilage condensation. Cell adhesion	8.00E-16	4
	Fc fragment of IgG binding protein (Fcgbp)	*Danio rerio*	Binding of sperm to zona pellucid. Cell adhesion	2.00E-07	1
	Nidogen 2 (Nid2)	*Danio rerio*	Cell adhesion. Cell-matrix adhesion	1.00E-42	1
	Ribosomal protein L29	*Ictalurus punctatus*	Cell proliferation. Cell-substrate adhesion	4.00E-41	5
	Vitronectin (Vtn)	*Taeniopygia guttata*	Cell adhesion. Cell-matrix adhesion	7.00E-56	1
Protein synthesis, transport and folding					
	Alpha 1 microglobulin/bikunin (Ambp)	*Xenopus tropicalis*	Protein maturation. Protein-chromophore linkage	4.00E-08	11
	Cyclophilin F	*Danio rerio*	Protein folding	4.00E-45	1
	DnaJ (Hsp40)	*Danio rerio*	Protein folding	1.00E-59	2

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Protein synthesis, transport and folding					
	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase (Ddost)	*Danio rerio*	Protein amino acid N-linked glycosylation via asparagines	1.00E-96	2
	Eukaryotic translation initiation factor 1b (Eif1b)	*Danio rerio*	Translational initiation	6.00E-38	2
	Eukaryotic translation initiation factor 6 (Eif6)	*Homo sapiens*	Integrin-mediated signalling pathway. Mature ribosome assembly	2.00E-82	1
	Eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	*Homo sapiens*	Transational regulation	3.00E-80	5
	Eukaryotic translation elongation factor 1 gamma (Eef1g)	*Protopterus dolloi*	Translational elongation	0	8
	Eukaryotic translation termination factor 1 (Etf1)	*Homo sapiens*	Translation. Translational termination	1.00E-05	2
	Heterogenous nuclear ribonucleoprotein K (Hnrnpk)	*Homo sapiens*	mRNA processing. RNA splicing	2.00E-31	1
	Mitochondrially encoded 16S rRNA (mt-Rnr2)	*Protopterus annectens*	Ribosomal large subunit assembly	1.00E-155	1
	RNA binding motif protein 8A (Rbm8a)	*Salmo salar*	Pre-mRNA processing	2.00E-80	1
	Ribosomal protein SA (Rpsa)	*Homo sapiens*	Translation	3.00E-113	2
	Ribosomal protein S3a (Rsp3a)	*Xenopus laevis*	Translation	4.00E-60	3
	Ribosomal protein S5 (Rps5)	*Mus musculus*	Translation	3.00E-38	2
	Small nuclear ribonucleoprotein polypeptides B (Snrpb)	*Salmo salar*	mRNA splicing	3.00E-59	2
Protein synthesis, transport and folding					
	Ribosomal protein S9 (Rps9)	*Protopterus annectens*	Translation	0	1
	Ribosomal protein S12 (Rps12)	*Taeniopygia guttata*	Translation	2.00E-39	1
	Ribosomal protein S15 (Rps15)	*Xenopus tropicalis*	Translation	4.00E-112	3
	Ribosomal protein S17 (Rps17)	*Mus musculus*	Translation	1.00E-103	4
	Ribosomal protein S20 (Rps20)	*Taeniopygia guttata*	Translation	1.00E-108	2
	Ribosomal protein S23 (Rps23)	*Homo sapiens*	Translation	6.00E-76	1
	Ribosomal protein S26 (Rps26)	*Solea senegalensis*	Translation	1.00E-77	1
	Ribosomal protein L3 (Rpl3)	*Xenopus laevis*	Translation	2.00E-107	4
	Ribosomal protein L4 (Rpl4)	*Protopterus dolloi*	Translation	2.00E-115	12
	Ribosomal protein L9 (Rpl9)	*Xenopus laevis*	Translation	3.00E-52	5

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Protein synthesis, transport and folding					
	Ribosomal protein L10 (Rpl10)	*Ictalurus punctatus*	Translation	4.00E-52	3
	Ribosomal protein L13a (Rpl13a)	*Salmo salar*	Translation	9.00E-13	4
	Ribosomal protein L15 (Rpl15)	*Mus musculus*	Translation	3.00E-84	2
	Ribosomal protein L19 (Rpl19)	*Protopterus dolloi*	Translation	4.00E-96	3
	Ribosomal protein L24 (Rpl24)	*Salmo salar*	Translation	2.00E-69	2
	Ribosomal protein L27 (Rpl27)	*Danio rerio*	Translation	1.00E-51	1
	Ribosomal protein L28 (Rpl28)	*Danio rerio*	Translation	6.00E-34	1
	Ribosomal protein L34 (Rpl34)	*Xenopus laevis*	Translation	4.00E-40	5
	Ribosomal protein Large P0 (Rplp0)	*Protopterus dolloi*	Translational elongation. Ribosome biogenesis	5.00E-62	1
	Ribosomal protein Large P2 (Rplp2)	*Ictalurus punctatus*	Transational elongation	3.00E-91	1
Structural					
	Tubulin, alpha 1 (Tuba1a)	*Xenopus laevis,*	Cell structure and motility. Intracelluar protein traffic. Chromosome segregation	2.00E-107	2
Signaling					
	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (Gnb2l1)	*Ornithorhynchus anatinus*	Intracellular signalling cascade. Protein localization	5.00E-57	2
Transcription					
	Reverse transcriptase/ribonuclease H/putative methyltransferase	*Danio rerio*	Reverse transcriptase. Proteolysis	7.00E-19	3
	Polyglutamine binding protein 1 (Pqbp1)	*Homo sapiens*	Regulation of transcription. DNA-dependent transcription	3.00E-07	4
Oxidative phosphorylation					
	Cytochrome P450 family 2 (Cyp2a)	*Danio rerio*	Electron transport	5.00E-28	2
Oxidative reduction					
	Deiodinase type III (Dio3)	*Neoceratodus forsteri*	Oxidation reduction. Hormone biosynthetic process	2.00E-33	2
	Dihydrodiol dehydrogenase (dimeric) (Dhdh)	*Danio rerio*	Metabolic process. Oxidation reduction	3.00E-26	1
Others					
	Angiotensinogen (Agt)	*Xenopus tropicalis*	Angiotension mediated vasoconstriction involved in regulation of systemic arterial blood pressure. Astrocyte activiation	4.00E-16	3

Table 7.3 Continued

Group	Gene	*Species*		E-value	No of clones
Others					
	Coiled-coil-helix-coiled-coil-helix domain containing 3 (Copb1)	*Mus musculus*	Unclassified	2.00E-06	2
	GEX Interacting protein family member (Gei-7)	*Danio rerio*	Unclassified	4.00E-103	1
	Serine (or cysteine) peptidase inhibitor, clade A, member 3A (Serpina3a)	*Xenopus laeivs*	Unclassified	2.00E-17	1
	Ribosomal protein L26 (Rpl26)	*Xenopus laevis*	Unclassifed	1.00E-39	5
	Transmembrane protein 11 (Tmem11)	*Taeniopygia guttata*	Unclassified	1.00E-38	1

Table 7.4. Known transcripts found in the reverse SSH library of the liver of *P. annectens* aestivated for 6 days in hypoxia.

Group	Gene (Symbol)	*Species*	Biological processes	E value	No of clones
Carbohydrate metabolism					
	Aldehyde dehydrogenase 3 family, member A2	*Taeniopygia guttata*	Other carbon metabolism	3.00E-63	2
	Fructose-bisphosphate, aldolase B (Aldob)	*Protopterus annectens*	Glycolysis. Metabolic process	8.00E-123	1
	Malate dehydrogenase 2, NAD (mitochondrial) (Mdh2)	*Taeniopygia guttata*	Carbohydrate metabolic process	1.00E-56	9
Other metabolism					
	Acetylserotonin O-methyltransferase (Asmt)	*Danio rerio*	Melatonin biosynthetic process	1.00E-07	1
	Inter-alpha trypsin inhibitor heavy chain 2 (Itih2)	*Taeniopygia guttata*	Hyaluronan metabolic process	6.00E-31	6
	Inter-alpha trypsin inhibitor, heavy chain 3 (Itih3)	*Danio rerio*	Hyaluronan metabolic process	4.00E-11	1
Complement					
	Complement component C3	*Protopterus aethiopicus*	Complement activation. G-protein coupled receptor protein signalling pathway	4.00E-62	6
Blood coagulation					
	Coagulation factor IX (F9)	*Xenopus tropicalis*	Blood coagulation. Proteolysis	4.00E-11	8
	Coagulation factor VII (F7)	*Xenopus tropicalis*	Blood coagulation. Proteolysis	5.00E-08	1
	Fibrinogen beta chain (Fgb)	*Taeniopygia guttata*	Blood coagulation. Platelet activation	9.00E-14	22
	Fibrinogen, A alpha chain (Fga)	*Xenopus laevis*	Blood coagulation	2.00E-65	2
	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1 (Serpinc1)	*Xenopus laevis*	Blood coagulation	7.00E-65	9
Iron, copper metabolism nd transport					
	Ceruloplasmin (Cp)	*Ornithorhynchus anatinus*	Copper ion transport	9.00E-22	11
	Haemoglobin, epsilon 1 (Hbe1)	*Xenopus tropicalis*	Oxygen transport	5.00E-14	4
	Transferrin (Trf)	*Oncorhynchus mykiss*	Iron transport	1.00E-57	29
Transport					
	ATP synthase, H+ transporting, mitochondrial F0 complex subunit C3 (Atp5g3)	*Danio rerio*	Ion transport. ATP synthesis coupled protein transport	1.00E-61	8
	Mitochondrial carrier, adenine nucleotide translocator	*Taeniopygia guttata*	Transport	4.00E-49	1
Response to Stimulus					
	Heat shock protein 20	*Ostertagia ostertagi*	Stress response. Response to heat	3.00E-04	19
Cell adhesion					
	Nidogen 2 (Nid2)	*Danio rerio*	Cell adhesion. Cell-matrix adhesion	1.00E-42	11

Table 7.4 Continued

Group	Gene (Symbol)	*Species*	Biological processes	E value	No of clones
Protein synthesis, transport, folding and modification					
	Mitochondrially encoded 16S ribosomal RNA gene (mt-Rnr2)	*Protopterus annectens*	Ribosomal large subunit assembly	1.00E-106	9
	Eukaryotic translation initiation factor 3, subunit E (Eif3e)	*Taeniopygia guttata*	Translation	1.00E-36	5
	Ribophorin I (Rpn1)	*Xenopus laevis*	Protein amino acid glycosylation	3.00E-59	7
	Ribosomal protein S3a (Rps3a)	*Taeniopygia guttata*	Translation	6.00E-108	9
	Ribosomal protein S17 (Rps17)	*Taeniopygia guttata*	Translation	2.00E-71	14
	Ribosomal protein L3 (Rpl3)	*Homo sapiens*	Translation	2.00E-74	1
	Ribosomal protein L7a (Rpl7a)	*Protopterus dolloi*	Ribosome biogenesis	1.00E-75	3
Structural					
	Crystallin, alpha A (Cryaa)	*Caiman crocodilus*	Actin filament organization. Camera-type eye development	1.00E-08	5
	Thymosin beta-like	*Taeniopygia guttata*	Actin cytoskeleton organization. Cytoskeleton organization	5.00E-12	2
	Vitalline membrane outer layer protein 1 homolog (Vmo1)	*Salmo salar*	Vitelline membrane formation	7.00E-10	1
mRNA processing					
	Y box protein 1 (Ybx1)	*Carassius auratus*	mRNA processing. In utero embryonic development	3.00E-41	8
Transcription					
	Polyglutamine binding protein (Pqbp1)	*Homo sapiens*	Regulation of transcription. DNA-dependent transcription	4.00E-05	10
	Transcription factor 7 (Sp7)	*Xenopus tropicalis*	Regulation of transcription form RNA polymerase II promoter. Osteoblast differentiation	5.00E-06	1
Cell cycle					
	Pelota homolog	*Xenopus tropicalis*	Cell cycle and division	6.00E-55	2
Nucleic acid binding					
	Heterogeneous nuclear ribonucleoprotein K (Hnrnpk)	*Xenopus tropicalis*	mRNA processing. RNA splicing	2.00E-78	9
Chromosome					
	A kinase (PRKA) anchor protein 8-like (Akap8l)	*Homo sapiens*	Unclassified	3.00E-33	20
Other					
	Alpha fetoprotein (Afp)	*Rattus norvegicus*	Progesterone metabolic process. Ovulation from ovarian follicle	2.00E-13	1
	Coiled-coil domain containing 124 (Ccdc124)	*Taeniopygia guttata*	Unclassified	1.00E-26	5

Table 7.4 Continued

Group	Gene (Symbol)	*Species*	Biological processes	E value	No of clones
Other					
	Fetuin beta (Fetub)	*Xenopus laevis*	Unclassified	7.00E-20	75
	Selenoporotein W2a	*Danio rerio*	Selenium binding	9.00E-29	33

7.4. Discussion

7.4.1. Six days of aestivation in normoxia – Forward library

*7.4.1.1. Up-regulation of OUC genes (***cps and ass***) and* **gs** *during the induction phase*

Using SSH, the author demonstrated that there were up-regulation of mRNA expression of OUC enzymes, particularly *cps* and *ass*, in the liver of *P. annectens* undergoing aestivation for 6 days in normoxia. However, it has been reported (Chapter 2; Loong et al., 2008b) that there were no changes in OUC enzyme activities in the liver of *P. annectens* after 6 days of aestivation in air (Loong et al., 2008b). This apparent controversy between high transcripts levels and low protein amount in these aestivating fish could be due to a decrease in the rate of translation. In anoxic turtles, protein synthesis is undetectable in liver and white muscle (Fraser et al., 2001) while translatable mRNA concentrations increase by 38% in the former and remain constant in the latter (Douglas et al., 1994), indicating that translation is the major site for the regulation of protein synthesis during metabolic depression (Pakay et al., 2003). Since *P. annectens* possesses CPS III which utilized glutamine as a substrate (Chapter 1; Loong at al., 2005), and glutamine is synthesized from glutamine synthetase, it is no coincidence that the regulation of *cps* occurred in association with an up-regulation of *gs* in fish aestivated in normoxia for 6 days.

7.4.1.2. Up-regulation of certain genes involved in fatty acid synthesis and transport

Fatty acid binding protein 1 (FABP) are intracellular carriers that transport fatty acids through cytoplasm, linking sites of fatty acid import/export (plasma membrane), internal storage (lipid droplets), and oxidation (mitochondria) (Storey and Storey, 2004). FABPs are crucial elements in successful hibernation because fatty acids are the primary fuel utilized throughout the winter by all organs (Storey and Storey, 2004). Stearoyl-CoA desaturase is a lipogenic enzyme that catalyzes the synthesis of monounsaturated fatty acids (FA). Deficiency of stearoyl-CoA desaturase activates metabolic pathways that promote FA β-

oxidation and decrease lipogenesis in liver (Dobrzyn et al., 2007). The up-regulation of several mRNAs encoding protein involved in lipoprotein metabolism indicates that during the induction phase of aestivation, there could be an increase in fatty acid synthesis. It was also shown that the fatty acid composition was significantly altered in terrestrial snail, Cepaea nemoralis, aestivating for 6 weeks (Stuart et al., 1998). This indicated that substantial membrane remodeling occurred in snail during aestivation. The up-regulation of several mRNAs encoding protein involved in lipoprotein metabolism indicates that during the induction phase of aestivation, there could be an increase in fatty acid synthesis or membrane remodeling.

*7.4.1.3. Up-regulation of mannan-binding lectin-associated serine protease (***masp***) could indicate lectin pathway as the preferred complement system during aestivation*

Mounting an immune response is energetically costly and must be performed at the expense of other physiological processes (Demas et al., 1997; Moret and Schmid-Hempel, 2000). Immune function in mammals diminishes during times of limited food availability and cold temperatures (Drazen et al., 2000). To the author's knowledge, the immune system of aestivating animals is not well-studied. The serum complement system is an important component of the immune system composed of more than 30 proteins involved in a cascade that protects an animal from a variety of pathogens (Sim and Laich, 2000; Ross, 1986; McAleer and Sim, 1993). The complement system mediates a chain reaction of proteolysis and assembly of protein complexes that results in the elimination of invading microorganisms (Walport, 2001a; Walport, 2001b). Three activation pathways (the classical, lectin and alternative pathways) and a lytic pathway regulate these events. In the classical pathway, C1q, a collagenous subcomponent of the first component (C1), binds to immunoglobulins within immune complexes, and its associated serine proteases, C1r and C1s, become activated. The complement cascade is initiated by the subsequent cleavage of C4 and C2,

followed by C3 activation. The resulting C3b fragment not only acts as an opsonin but also leads to the membrane attack complex formation in the lytic pathway. In innate immunity, a complex composed of a recognition molecule (lectin) and serine proteases, termed the mannose-binding lectin (MBL)-associated serine protease (MASP), activates C4 and C2 upon binding to carbohydrates on the surface of microorganisms via the lectin pathway. This binding occurs in the absence of immunoglobulin (Fujita et al., 2004). Results from this study indicate that an up-regulation of mannan-binding lectin-associated serine protease (*masp*) occurred in fish aestivated in normoxia for 6 days. Hence, *P. annectens* appeared to utilize the lectin pathway for protection against pathogens during aestivation. The classical pathway was not a desirable choice, probably because of the need to synthesize immunoglobins.

7.4.1.4. Up-regulation of tissue factor pathway inhibitor suggested a suppression of clot formation during aestivation

Tissue factor pathway inhibitor (*tfpi*) of coagulation was up-regulated in fish aestivating in normoxia. Injury to blood vessel walls exposes tissue factor (TF) to circulating Factor VII, and TF forms a complex with the active (a) form of FVII (TF-FVIIa) that induces a conformational change in the protease domain of and activates Factor VIIa (Price et al., 2004). In turn, this protease activates Factor IX and X which then go on to simulate the conversion of prothrombin to thrombin. The protein, TFPI, is an inhibitor of the TF-FVIIa complex and the main regulator of the tissue factor pathway (Price et al., 2004). Hence, TFPI has an important anticoagulant action. The up-regulation of *tfp*1 in *P. annectens,* revealed a suppression of spontaneous clot formation during 6 days of aestivation in normoxia.

7.4.1.5. Aestivation in normoxia resulted in the up-regulation of genes related to iron metabolism

Iron is an essential element required for the growth and survival of most organisms, since it is involved in many cellular metabolic pathways and enzymatic reactions. However, iron is also toxic to the cells, when in excess (Cammack et al., 1990; Linn, 1998; Harrison and Arosio, 1996). Since many organisms, including fish, lack a regulated pathway for iron excretion, iron balance is maintained by a tight regulation of absorption in the intestine, responding to the level of body iron stores and to the amount needed for erythropoiesis and other functions (Neves et al., 2009). Transferrin is one of the major serum proteins in eukaryotes and plays a crucial role in iron metabolism. It is mainly synthesized in the liver and secreted into the blood. Under normal conditions, most of the iron in the blood plasma is bound to transferrin, and iron-transferrin complexes enter the cells via a transferrin receptor-mediated endocytic pathway. Transferrin also has a close relation with the immune system. It binds to iron, creating an environment with low levels of iron, where few microorganisms can survive and prosper (Neves, 2009). Ferritin is the main iron storage protein in both eukaryotes and prokaryotes; it keeps iron in a soluble and non-toxic form (Chasteen, 1998; Harrison and Arosio, 1996; Thei, 1990). In its iron-free form, most ferritin complexes consist of 24 subunits of heavy (H) and light (L) chain, with molecular masses of 21 and 19 kDa, respectively. Synthesis of ferritin is known to be induced when iron is available, whereas under conditions of iron deprivation, ferritin synthesis is repressed (Zahringer et al., 1976; Torti and Torti, 2002; Lieu et al., 2001). Also, up-regulation of ferritin is observed in oxidative stress (Orino et al., 2001) and inflammatory conditions (Torti and Torti, 2002; Torti et al., 1988; Rogers et al., 1990), being induced by exposure to lipopolysaccharides and proinflammatory cytokines, which suggests a link to immune response. Delaney et al. (1976) previously reported that during the first month of aestivation, *Protopterus aethiopicus* develops an increase in circulating neutrophils. These neutrophils were transient and subsided within the next 60 days. This sequence is consistent with the idea of stress or local inflammation at the start of aestivation. The up-regulation of transferrin and ferritin in this

study could be due to two possible reasons. First, both genes were up-regulated due to oxidative stress and inflammatory conditions during the early phase of aestivation. Secondly, transferrin and ferritin were induced due to a high turnover rate of free and bound iron as a result of increased synthetic rate of haemoglobin in general or certain type of haemoglobins. Up-regulation of certain haemoglobin genes during 6 days of aestivation in normoxia were observed in this study.

7.4.1.6. Up-regulation of ceruloplasmin could be due to tissue injury or inflammation

Plasma copper is distributed among three major constituents comprising two pools, between which it does not appear to be exchangeable (Marceau and Aspin, 1972; Sternlieb et al., 1961). Ceruloplasmin represents a tightly bound pool that accounts for at least 90% of the total plasma copper in most species (Gubler et al., 1953; Henkin, 1974). Copper secreted from hepatocytes (parenchymal cells) is principally in the form of ceruloplasmin. Extrahepatic copper is probably presented to tissues in this form. Owen (1965) and Campbell et al. (1981) demonstrated that copper uptake into tissues was closely related to release of ceruloplasmin from the liver. Ceruloplasmin synthesis and/or secretion is altered by inflammation, hormones, and copper. It is generally recognized that plasma concentrations of acute-phase globulins, including ceruloplasmin, increase with tissue injury, localized acute inflammation, and chronic inflammatory diseases (Cousins, 1985).

7.4.1.7. Up-regulation of two types of haemoglobins

Haemoglobin epsilon and gamma were up-regulated in normoxic condition. Delaney et al. (1976) reported the presence of 4 electrophoretically distinct types of haemoglobins in *Protopterus aethiopicus*. These 4 types of haemoglobins (fraction I, II, III and IV) were presented in both the aquatic and aestivating lungfish. Delaney et al. (1976) reported a relative increase in the amounts of types II and IV, within 3 months of aestivation and these

two types persisted for the remaining 13 months of aestivation. Unfortunately, there was no other attempt made to identify these fractions. It is possible that haemoglobin gamma and epsilon coded for the two types of haemoglobin (fraction II and IV) that were reported by Delaney et al. (1976).

7.4.1.8. Increased translation for synthesis of selected proteins

Protein synthesis is a major energy expense in cells and it will utilize a notable amount of cellular energy, which is mainly consumed in the process of translation. It is estimated that up to 50% of the cellular energy (depending on the organism) is consumed during translation (Mathews et al., 2000; Warner, 1999; Rudra and Warner, 2004). Due to the high usage of cellular energy, translation of genes becomes more selective in order to conserve energy but at the same time, allow critical functions to carry on. Gingas et al. (1999) reported that under stress condition, message selection for translation changes to favour only those that contain an internal ribosome entry site (IRES). It is possible that up-regulation of the ribosomal proteins during aestivation in normoxia is for translating selected messages that codes for proteins that allow the fish to survive during aestivation. However, further studies are required to verify this proposition.

7.4.2. Six days aestivation in normoxia - Reverse library (down-regulation)

7.4.2.1 Down-regulation of genes related to carbohydrate metabolism

Glyceraldehyde-3-phosphate dehydrogenase, enolase and lactate dehydrogenase are enzymes involved in glycolysis while fructose-1,6-bisphosphatase and enolase are enzymes involved in gluconeogenesis. These results indicate for the first time that a reduction in glycolytic rate could be an important adaptive process during the late induction phase of aestivation. A possible role for the transcriptional activation of these genes would be to increase the capacity for allosteric regulation of the glycolytic and gluconeogenesis pathways

during hypoxia. A few studies proposed that in the hibernating ground squirrels, there was a regulation between anaerobic glycolysis and gluconeogenesis. Tashima et al. (1970) utilized radioglucose tracing on active, hibernating and arousing ground squirrels, and revealed that noncarbohydrates are the primary source of oxidative energy during hibernation. Gluconeogenic activity was demonstrated to be enhanced during hibernation (Burlington and Klain, 1967; Green et al., 1984). Studies had shown that there was no decrease in tissue glycogen content in the tissue of aestivating *P. dolloi* during the maintenance phase of aestivation (Frick et al., 2008a; Janssens and Cohen, 1968b), which lead the author to suggest that the lungfish could have undergone "glycogen sparing". Under "glycogen sparing", glycogen reserves must be maintained by gluconeogenesis from protein-derived amino acid (Hochachka and Guppy, 1987). The increase in rate of urea synthesis during the induction phase of aestivation indicate that amino acid catabolism was not suppressed to a great extent and some of the carbon chains released during deamination could be channeled into glycogen formation.

7.4.2.2. Further evidence supporting lectin pathway for innate immunity during aestivation

Complement component 1q belongs to the subcomponent of the first component (C1) in the classical pathway, which binds to immunoglobulin within immune complexes. The down-regulation of complement component 1q further supports the preference toward lectin pathway as a choice for innate immunity in *P. annectens* during aestivation.

7.4.2.3. Aestivation in normoxia resulted in decrease in clot formation

SERPINC1 (antithrombin) inhibits thrombin and, thereby, also blocks feedback activation of the cascade by thrombin (Storey, 2004). These genes are involved in clot formation. Together with the findings for up-regulation of tissue factor pathway inhibitor, these results showed that 6 days of aestivation in normoxia resulted in a decrease in clot

formation. Ahmad et al. (1979) demonstrated a prolongation of whole blood clotting time in hibernating frog (*Rana tigrina*). They concluded that retarded blood clotting at lower temperature may play an important protective role against intravascular thrombosis. Several other studies also demonstrated prolonged clotting time in hibernating animals (Svihla et al., 1951; Pivorun and Sinnamon, 1981). Since, the heart rate of African lungfish, *Protopterus aethiopicus*, drops from 22-30 beats/min before aestivation to 12-17 beats/min by the end of 1-1.5 months in the mud (Delaney et al., 1974), it is probable that a severe decrease in the rate of blood flow would have occurred. Thus, any mechanism that can prevent the formation of a thrombosis during the inactive aestivating fish would be of considerable survival value.

7.4.2.4. Reduction in translation due to down-regulation of genes coding for ribosomal protein and translational elongation factor

Recently, Icardo et al. (2008) revealed that aestivation appears to trigger an increase in transcriptional and synthetic myocardial activities in the heart of the lungfish. In addition, Ojeda et al. (2008) demonstrated structural modifications in all components of the renal corpuscle of aestivating *P. dolloi*. A large number of genes coding for ribosomal proteins (40S and 60S subunits) as well as translational elongation factors were up- and down-regulated in 6 days aestivation in normoxia. Together with the findings from other studies, it is probable that aestivation could involve simultaneous synthesis and degradation of different proteins which was accounted for by the up- and down-regulation of ribosomal protein and translational elongation factor.

7.4.3. Six days of aestivation in hypoxia – Similarities to normoxia

*7.4.3.1. Up-regulation of OUC genes (***cps** *and* **ass***) and* **gs** *in hypoxia*

Similar to normoxia, there were increases in mRNA expression of *cps*, *ass*, and *gs* in the liver of *P. annectens* after 6 days of aestivation in hypoxia. Hence, it can be concluded that upregulation of OUC capacity and urea synthesis was essential to the induction phase of aestivation in *P. annectens*. Hypoxia prescribed a decrease in efficiency of ATP production (Chapter 3; Loong et al. 2008a). If increased urea synthesis is non-essential to the induction of aestivation, the mRNA and protein expression of OUC enzymes should be suppressed to conserve metabolic energy. Since, the magnitude of up-regulation of mRNA expression of *cps*, *ass* and *gs* involved might differ between normoxia and hypoxia and since such information was not reflected by SSH, it was important to further examine this phenomenon by qPCR (Chapter 5).

7.4.3.2. Up-regulation of genes related to fatty acid synthesis, complement and blood coagulation in both normoxia and hypoxia

Both normoxia and hypoxia up-regulated the expression of stearoyl-CoA desaturase. Hence, aestivation in normoxia or hypoxa might involve increased fatty acid synthesis.

Similar changes in gene expression of complement activation and blood coagulation occur in fish aestivating in hypoxia. Both aestivating in normoxia and hypoxia prescribe the use of lectin pathway for protection against pathogens and suppress clot formation.

7.4.3.3. Up-regulation of genes related to iron and copper metabolism in hypoxia

Transferrin and ferritin were up-regulated in normoxia and hypoxia. Similar to earlier explanations, both genes could be up-regulated due oxidative stress or inflammatory conditions that could happen during the early phase of aestivation. Haemoglobin epsilon and gamma were also up-regulated in hypoxia. It is likely that up-regulation of transferrin and ferritin were due to the need to synthesize haemoglobin.

Ceruloplasmin increased after tissue injury, localized acute inflammation and chronic inflammatory diseases (Cousins, 1985). Ceruloplasmin can scavenge oxygen-centre free radicals which aggravate inflammation. An experiment conducted by Denko (1979) on ceruloplasmin revealed that ceruloplasmin injection resulted in reduction of experimental inflammation. This showed that ceruloplasmin is a possible protective mechanism against inflammation during aestivation. Both normoxia and hypoxia resulted in an up-regulation of ceruloplasmin expression in the liver. During aestivation, the fish is immobilized and its heart rate and blood flow decrease. These could lead to inflammation in the lungfish. It is possible that the increase in ceruloplasmin would have some protective effects on aestivating *P. annectens*.

7.4.3.4. Up-regulation of genes related to ribosomal protein and translational elongation factor in both normoxia and hypoxia

Similar to aestivation in normoxia, the up- and down-regulation of ribosomal protein and translational elongation factor could involve simultaneous protein synthesis and degradation of liver cells.

7.4.4. Differences from normoxia

7.4.4.1. Up-regulation of genes related to carbohydrate metabolism in hypoxia but not in normoxia

Fish exposed to hypoxic stress may either reduce metabolic rate to match the reduced supply of energy, or maintain metabolic rate and increase anaerobic metabolism (glycolysis) in order to make up the ATP deficit (Johnston, 1975; Nilsson, 1990; Nikinmaa, 2002). Indeed, 6 days of aestivation in hypoxia induced an up-regulation of genes related to anaerobic metabolism in *P. annectens*. These genes included aldoase A, aldolase B, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase. Therefore, it can be

concluded that *P. annectens* might at least partially undergo fermentative glycolysis during the induction phase of aestivation in hypoxia. This must be regarded as a response to hypoxia and not intrinsic to the aestivation process because 6 days aestivation in normoxia prescribed a down-regulation of genes (aldolase B, glyceraldehydes-3-phosphate dehydrogenase, lactate dehydrogenase, and enolase) from anaerobic metabolism in the liver of *P. annectens* instead. Six days aestivation in hypoxia also induced genes related to gluconeogenesis. The expression of fructose-1,6-bisphosphatase, fructose-2,6-bisphosphatase, aldolase and phosphoenolpyruvate carboxykinase were up-regulated during 6 days aestivation in hypoxia. These results reiterate the importance of maintaining a certain level of glycogen in the liver of *P. annectens* during the induction phase of aestivation. They also indicate that increased glycolysis could be compensated by an increase in gluconeogenesis and glycogen synthesis.

Six days of aestivation in hypoxia up-regulated hepatic lipase which was not observed in normoxia. Hepatic lipase hydrolyses triglycerides and release glycerol which may be channeled into the gluconeogenic pathway to provide substrate for glycogen synthesis or into part of the glycolytic pathway for energy production.

From these findings, it was revealed that hypoxia prescribes differences in gene expression of carbohydrate metabolism at least during the induction phase (i.e. 6 days) of aestivation in *P. annectens*. However, it is unclear whether long-term maintenance of aestivation would result in similar gene expression. Thus, future studies should be done to verify the gene expression throughout the different phases of aestivation in different conditions.

7.4.4.2. Up-regulation and down-regulation of genes in the same condition

Ferritin heavy chain and some ribosomal protein were up- and down-regulated in normoxic condition. Ceruloplasmin, complement component and some ribosomal proteins 3

were up- and down-regulated in hypoxic condition. This would indicate that these few genes were false positive or they represented different isoforms of the same genes. Further study is required to verify this.

7.5. Summary

This study aimed to confirm through SSH that mRNA expression of OUC enzymes, especially CPS III, were up-regulated in the liver of *P. annectens* undergoing 6 days of aestivation, i.e. during the transition between the induction phase and the initial maintenance phases of aestivation, in normoxia or hypoxia. In addition, the author aimed to test the hypothesis that, besides certain OUC enzymes, the gene expression of some other enzymes involved in amino acid and carbohydrate metabolism in the liver would be differentially affected by aestivation in normoxia and aestivation in hypoxia. Using SSH, it was demonstrated that expressions of genes related to urea synthesis, i.e. *cps*, *ass* and *gs* were up-regulated in *P. annectens* after 6 days of aestivation in air or in hypoxia. These results support the conclusions that urea synthesis in this lungfish involved CPS III which uses glutamine as a substrate, and that increased urea synthesis, although energy intensive, is an important adaptive response of aestivation, even during perturbation of energy metabolism in hypoxia. Results obtained from SSH also revealed that several gene clusters were up- or down-regulated during the induction phase of aestivation. Overall, they indicate that the induction of aestivation involved probably the prevention of clot formation, activation of the lectin pathway for complement activation, conservation of minerals (e.g. iron and copper) and increased production of certain types of haemoglobin. There could also be simultaneous increased in protein degradation and protein synthesis, hinting at the importance of reconstruction of protein structures in preparation for aestivation. More importantly, 6 days of aestivation in hypoxia led to up-regulation of genes related to anaerobic energy metabolism, some of which were down-regulated in fish aestivated in normoxia for 6 days. Hence, it can be concluded that increased fermentative glycolysis was a response to hypoxia and not intrinsic to the aestivation process.

8. Chapter 5:

Determination of mRNA expression of carbamoyl phosphate, argininosuccinate synthetase, glutamine synthetase and glutamate dehydrogenase in the liver of *Protopterus annectens* undergoing different phases of aestivation in various conditions

8.1. Introduction

Protopterus annectens can aestivate inside a cocoon made of dried mucus in air or burrow into the mud and aestivate in a subterranean cocoon (Chapter 2; Loong et al., 2008a). It is ureogenic and possesses a full complement of ornithine-urea cycle (OUC) enzymes, including carbamoyl phosphate synthetase III (CPS III) which require glutamine as a substrate, in the liver (Chapter 1; Loong et al., 2005). During aestivation, *P. annectens* avoids ammonia toxicity through an increase in urea synthesis and a suppression of ammonia production (Chapter 2; Loong et al., 2008b). Fish aestivating in mud had a lower dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, than fish aestivating in air (Chapter 2; Loong et al., 2008b). In addition, different adaptive responses were exhibited by fish aestivating in normoxia and in hypoxia (Chapter 3; Loong et al., 2008a). These results indirectly support the proposition that reduction in nitrogen metabolism, and probably metabolic rate, did not occur simply in association with aestivation (in normoxia) but responded more effectively to a combined effect of aestivation and hypoxia.

Indeed, such a proposition was confirmed by comparing and contrasting results obtained on up- and down-regulations of gene expressions in the livers of fish after 6 days of aestivation (i.e. at the onset of formation of a complete mucus cocoon) in normoxia and those of fish after 6 days of aestivation in hypoxia (2% O_2 in N_2) using suppression subtractive hybridization (SSH) PCR (Chapter 4). More importantly, results obtained through SSH PCR revealed that mRNA expression of two OUC enzymes, carbamoyl phosphate synthetase (CPS) and argininosuccinate synthetase (ASS), were consistently up-regulated in fish aestivating in normoxia and in hypoxia. Since *P. annectens* and other African lungfishes are known to possess CPS III instead of CPS I (Chew et al., 2003b; Loong et al., 2005), and since CPS III requires glutamine as a substrate, it was therefore no coincidence that glutamine synthetase (GS) was also up-regulated in fish undergoing aestivation in both conditions. In

order to determine the exact magnitude of up-regulation (i.e. fold-changes) in mRNA expression of these three enzymes, specific quantitative RT-PCR (qPCR) primers were designed based on the partial gene fragments obtained through SSH and this study was undertaken to examine their mRNA expression in the liver of *P. annectens* at different phases of aestivation under various experimental conditions using qPCR.

Surprisingly, glutamate dehydrogenase (*gdh*) was not among the 1000 gene fragments analyzed for up- or down-regulation of gene expression in the liver of *P. annectens* after 6 days of aestivation (i.e. the end of the induction phase). However, GDH could be crucial to amino acid metabolism (see Chapter 3) during other phases of aestivation, for instance, during the arousal phase when it becomes essential to mobilize internal protein resources for energy supply and regeneration of certain tissues and organs before the initiation of feeding. Therefore, a special effort was made in this study to obtain a partial sequence of GDH from the liver of *P. annectens*. Subsequently, based on the partial sequence obtained, qPCR primers were designed to determine the mRNA expression of *gdh*.

There were three sets of experiments in this study. In experiment A, efforts were made to determine the mRNA expression of *cps*, *ass*, *gs* and *gdh* in the liver of fish during the maintenance phase (12 or 46 days) of aestivation in air versus in mud. In experiment B, the mRNA expression of these four enzymes in the liver of fish undergoing induction (3 or 6 days) and early maintenance (12 days) phases of aestivation in normoxia were examined and compared with those in fish undergoing similar phases of aestivation in hypoxia. Finally, in experiment C, an attempt was made to evaluate the mRNA expression of these four enzymes in the liver of fish undergoing the induction (3 or 6 days), early or prolonged maintenance (12 days or 6 months) and arousal (1, 3 or 6 days of recovery without food supply in water after 6 months of aestivation) phases of aestivation in air (normoxia) as compared with the freshwater control.

Overall, four hypotheses were tested in this study. First, mRNA expression of *cps*, *ass* as OUC enzymes and *gs*, the enzyme that supply glutamine to CPS III, would be up-regulated during the induction phase and earlier maintenance phase (3-12 days) of aestivation in air, in mud and in hypoxia, and the magnitude of increase in expression of these three enzymes were comparable under all three conditions. Second, mRNA expression of *cps* III, *ass* and *gs* would return to control level or be suppressed during the early and prolonged maintenance phase of aestivation in air (40 days to 6 months, respectively) or in mud (40 days). Third, mRNA expression of *gdh* would be up-regulated during the induction phase (3-6 days) of aestivation in hypoxia but not in normoxia (refer to Chapter 3). Fourth, mRNA expression of *gdh* would be up-regulated after the fish was aroused from six months of aestivation and returned to water.

It was hoped that results obtained would provide definitive evidence to support the proposition that physiological phenomena involved in aestivation, e.g. increased urea synthesis, could vary between different phases of aestivation and differ between normoxic and hypoxic conditions. This would suggest to future researchers working on aestivating African lungfish to take a cautious approach with those information available in the literature, which do not specify the degree of hypoxia and the exact phase or period of aestivation involved. It would also prompt future researchers to specify the degree of hypoxia and the phase of aestivation that the lungfish specimens have been exposed to during the experiment.

8.2. Materials and methods

8.2.1. Fish

Protopterus annectens (80-120 g body mass) were imported from Central Africa through a local fish farm in Singapore. Specimens were maintained in plastic aquaria filled with dechlorinated water, containing 2.3 mmol l^{-1} Na^{+}, 0.54 mmol l^{-1} K^{+}, 0.95 mmol l^{-1} Ca^{2+}, 0.08 mmol l^{-1} Mg^{2+}, 3.4 mmol l^{-1} Cl^{-} and 0.6 mmol l^{-1} HCO_3^{-}, at pH 7.0 and at 25°C in the laboratory, and water was changed daily. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 1 month. During the adaptation period, fish were fed frozen fish meat.

8.2.2. Experiment A: Exposure of fish to 12 days or 46 days of aestivation in air or in mud and collection of samples

It has been reported recently that increases in salinity and changes in ionic composition in the external medium could induce a decrease in ammonia production, which is an important facet of aestivation, in *P. dolloi* (Ip et al., 2005b). So, following the procedure of Chew et al. (2004) for *P. dolloi*, *P. annectens* were induced to aestivate at 27-29^{o}C and 85-90% humidity individually in plastic tanks (L29 cm x W19 cm x H17.5 cm) containing 15 ml dechlorinated tap water (made 0.3‰ with seawater). It took approximately 6 days for the fish to be encased in a brown dried mucus cocoon. In this study, these 6 days were counted as part of the aestivation period. So, for a fish aestivated for 12 days, it would have spent at least 6 days within the dried mucus cocoon. Similarly, a fish aestivated for 46 days would have been in the cocoon for approximately 40 days.

Dried mud collected from the bottom of freshwater ponds was purchased from Hua Hing Trading Co. (Singapore). The dried mud was soaked in dechlorinated tap water for at least 2 days, and mixed into a thick paste (approximately 30% water content) by hand. Artificial muddy substrata (19 kg dry mass) with a minimum depth of 15 cm were made in

plastic tanks (L29 cm x W19 cm x H17.5 cm). Fish (one per tank) were allowed to bury at liberty into mud, which took 2-12 h. A small amount of water (approximately 100 ml) was evenly spread on to the surface of the mud every 4-5 days to prevent the surface mud from drying up and cracking.

On days 0 (freshwater control), 12 and 46 of aestivation in air or in mud, fish were killed with a strong blow to the head. The liver was quickly excised and freeze-clamped with aluminium tongs pre-cooled in liquid nitrogen. Frozen samples were kept at -80^{o}C until analysis.

8.2.3. Experiment B: Exposure of fish to 3, 6, or 12 days of aestivation in normoxia or hypoxia (2% O_2 in N_2) and collection of samples

Normoxic fish were exposed individually to terrestrial conditions and allowed to enter into aestivation at 23^{o}C in air-tight plastic containers (17.5 cm x 11.5 cm x 5 cm, length x width x height) containing 20 ml of water. The head space of boxes was flushed continuously (50 ml min^{-1}) with air (20.9% O_2 in N_2) for 12 days. Hypoxic fish underwent aestivation in similar plastic containers but they were flushed with 2% O_2 in N_2 instead. Under standard laboratory conditions, the experimental fish would secrete mucus during the first few days, and the mucus would slowly dry up between day 6 and day 7 to form a mucus cocoon. Therefore, three major time points were defined in this study, i.e. day 3 (induction phase of aestivation), day 6 (induction phase of aestivation) and day 12 (maintenance phase of aestivation). Fish were killed on days 3, 6 or 12 with a strong blow to the head. Liver were sampled and kept at -80°C until analysis.

8.2.4. Experiment C: Exposure of fish to induction phase, early maintenance phase, and prolonged maintenance phase of aestivation and followed by arousal from aestivation.

Protopterus annectens were induced to aestivate at 27-29^{o}C and 85-90% humidity individually in plastic tanks (L29 cm x W19 cm x H17.5 cm) containing 15 ml dechlorinated tap water (made 0.3‰ with seawater). It took approximately 6 days for the fish to be encased in a brown dried mucus cocoon. In this study, these 6 days were counted as part of the aestivation period. So, for a fish aestivated for 12 days, it would have spent at least 6 days within the dried mucus cocoon. The fish were allowed to aestivate for 6 months. After 6 months of aestivation, some of the fish were aroused by adding 200 ml of water into the tank and breaking up the cocoon manually. After a few minutes, the fish would swim sluggishly in the water; another 800 ml of water will be added to cover the fish. Fish were killed on 3 or 6 days of induction phase of aestivation, after 12 days (early maintenance), or 6 months (prolonged maintenance) of maintenance phase of aestivation, or after 1 day, 3 days, or 6 days of arousal phase of aestivation (in freshwater with food after arousal from 6 months of aestivation).

8.2.5. Extraction of total RNA

Total RNA was extracted from the liver, using chaotropic extraction protocol described by Whitehead and Crawford (2005). Frozen liver tissues were homogenized using an electric homogenizer (Pro Scientific Inc., Oxford, CT, USA) in 400 µl chaotropic buffer (4.5 M guanidinium thiocyanate, 2% N-lauroylsarcosine, 50 mM EDTA (pH 8.0), 25 mM Tris-HCl (pH 7.5), 0.1 M β-mercaptoethanol, 0.2% antifoam A). All reagents were from Sigma. Sodium acetate (2 M, pH 4.0) was added to the homogenate, followed by 400 µl acidic phenol (pH 4.4), and 200 µl chloroform/isoamyl alcohol (23:1). The mixture was kept at 4°C for 10 min then centrifuged at 4°C at 10,000 x*g* for 20 min. Supernatant (400 µl) was removed and combined with 400 µl isopropanol, stored at -20°C overnight. Supernatant was centrifuged at 10,000*g* for 30 min at 4°C. The remaining RNA pellet was rinsed twice with 500 µl of 70% ethanol, then further purified using the Qiagen RNeasy Mini Kit (Qiagen Inc.,

CA, USA) following the manufacturer's protocols. RNA quality was checked electrophoretically by running 1 µg of RNA in 1% agarose gel. RNA quantification was done spectrophotometrically using Hellma traycell (Hellma GmbH & Co. KG, Müllheim, Germany).

8.2.6. Obtaining **gdh** *fragment from PCR*

Total RNA (1 µg) was reverse transcribed into cDNA using RevertAid first strand cDNA synthesis kit (Fermentas Int. Inc., Burlington, Canada) using oligo dT primer according to the manufacturer's protocol. PCR was performed on these cDNAs using degenerate primers (Forward: 5'-ATGTACMGRYRYYTSGGRGA-3'; Reverse: 5'-CRTGRTTHAGRTTCTTBAGC-3'). Forward primer was obtained from Hirata et al. (2003). Reverse primer was designed based on identifying highly conserved regions from multiple alignment of *gdh* mRNA sequences available in the Genbank (http://www.ncbi. Nlm.nih.gov/Genbank/) from fish species (*Danio rerio, Tribolodon hakonensis, Onchorhynchus mykiss,* and *Salmo salar*) and other vertebrate species (*Xenopus laevis, Xenopus tropicalis, Rattus norvegicus,* and *Homo sapiens*). Each PCR was carried out in 25 µl reaction volumes, containing 2.5 µl of 10X Dreamtaq Buffer, 0.5 µl of dNTPs (10 µM), 0.5 µl of $MgCl_2$ (25mM) , 0.125 µl of Dreamtaq (5 unit/µl) (Fermentas Int. Inc., Burlington, Canada), 1.25 µ1 of forward primer (10 µM), 1.25 µl of reverse primer (10 µM) and 0.5 µl of cDNA template. PCR was performed in a Biorad Peltier thermal cycler (Biorad laboratories Inc., Hercules, CA, USA). The following thermocycling program was used: 95°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 42°C for 30 sec, 72°C for 1.5 min and 1 cycle of final extension of 72°C for 10 min.

PCR products were separated by 1% agarose gel electrophoresis and band of expected size was excised from the gel. The PCR product was purified from the gel slice by

centrifugation method using Wizard SV gel and PCR clean-up system kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The concentration of gel purified PCR product was determined spectrophotometrically using Hellma traycell (Hellma GmbH & Co. KG, Müllheim, Germany).

About 80-100 ng of purified PCR product was used in Bigdye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) with 2 μM of gene specific primers. The following thermocycling conditions were used: 96°C for 2 min, following by 25 cycles of 96°C for 30 sec, 55°C for 15 sec, 60°C for 4 min. Excess fluorescent nucleotides and salts were removed from the sample by ethanol/sodium acetate precipitation. Specifically, 1μl of 3 mol l^{-1} sodium acetate (pH 4.6) and 25 μl of 95% ethanol were mixed with the reaction mix and incubated for 15 min before centrifugation at 14,000 rpm for 15 min. The resulting pellet was washed with 75% ethanol twice and air-dried for 5 min. The dried sample was resuspended in Hi-Di Formamide for loading into the Prism™ 3130XL sequencer (Applied Biosystems, CA USA).

8.2.7. Designing primers for real-time PCR

Primers were designed against the 5 selected genes (Table 8.1). *cps*, *ass*, *gs* and actin were designed against the fragment obtained from SSH. *gdh* was designed against the fragment obtained from degenerate primers. All primer pairs produced amplicons of the predicted size. To confirm amplification specificity, the PCR products from each primer pairs was subjected to agarose gel electrophoresis. The bands were excised from the gel. The PCR products were then purified from the gel by centrifugation method using Wizard SV gel and PCR clean-up system kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The purified PCR products was quantified and used in Bigdye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA USA) as described above.

8.2.8. cDNA synthesis for real-time PCR

Prior to reverse transcription, total RNA from all samples intended for real-time PCR were subjected to DNase treatment using deoxyribonuclease I in accordance to the manufacturer's protocol (Sigma-Aldrich, St-Louis, MO, USA). Each sample was reverse transcribed by RevertAid first strand cDNA synthesis kit (Fermentas Int. In., Burlington, Canada) from 1 µg of total RNA using random hexamer according to the manufacturer's protocol.

8.2.9. Relative quantification by real-time PCR

Real-time PCR reactions were performed in duplicates by using 10 ng of cDNA template, 0.3 µM of each primer, and 5 µl of iTaq SYBR Green supermix containing 3 mM $MgCl_2$, 200 µM of each dNTPs, 0.25 units of iTaq DNA polymerase, and SYBR Green I dye in a volume of 10 µl. Reactions were analyzed on an iQ5 Real-Time PCR Detection system (Biorad laboratories Inc., Hercules, CA, USA) and cycling conditions were as follows: 3 min at 95ºC for antibody-mediated hot-start iTaq DNA polymerase activation and 45 cycles for the melting (5 sec, 95ºC) and annealing/extension (10 sec, 60ºC) steps. Runs were followed by melt curve analysis increasing from 55 to 95ºC in 0.5ºC increments. No primer dimers were detected. Relative standard curves were generated by serial diluting cDNA from freshwater fish liver in 5-fold. These relative standard curves were then used to determine the amplification efficiencies of all the genes. The amplification efficiencies of all the genes tested were ranged between 90% to 100%. For the $\Delta\Delta C_t$ calculation to be valid, the amplification efficiencies of the target and reference gene must be approximately equal (Livak and Schmittgen, 2001). A plot of the log cDNA dilution versus ΔC_t was made and the absolute value of the slope is closed to zero. $\Delta\Delta C_t$ calculation for relative quantification of

target then was applied to calculate the fold change in gene expression. All data were normalized to the abundance of actin mRNA.

8.2.10. Statistical analyses

Results were presented as means ± S.E.M. Student's t-test and one-way analysis of variance (ANOVA) followed by multiple comparison of means by the Tukey test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $p<0.05$.

Table 8.1. Primer sequences used for real-time PCR.

Genes	Primer name	Primer sequence	PCR product size (bp)
cps	CPS Fc	5’-TTGGTTACCCAGTGATGATCCGA-3’	172
	CPS Rc	5’-CACTTCATACTCCACCTCCTTCC-3’	
ass	ASS Fc	5’-CATGGAGTATGGATGCTAACCT-3’	197
	ASS Rc	5’-GTACTGTCTTATCGTTGAGATTGG-3’	
gs	GS FC	5’-GTGACATGTACCTCATCCCA-3’	187
	GS RC	5’-TACTCCTGCTCCATGCCAAACCA-3’	
actin	Actin F2	5’-CATACTGTGCCCATTTATGAAGGT-3’	75
	Actin R2	5’-CAAGTCACGGCCAGCTAAATC-3’	
gdh	Gdh FB	5’-TTTCATTGGTCCTGGCATTG-3’	100
	Gdh RB	5’-AATGTCCCATTGTGTTGGCATA-3’	

8.3. Results

8.3.1. mRNA expression of cps *III,* ass*,* gs *and* gdh *in the liver of fish during the maintenance phase (12 or 46 days) of aestivation in air versus in mud*

There were no significant differences in the Ct values and fold-changes in mRNA expression of the reference gene, *actin*, in the liver of *P. annectens* kept in freshwater (control), fish after 12 or 46 days of aestivation (inclusive of 6 days of induction) in air and fish aestivating in mud for 12 or 46 days (Table 8.2).

In comparison, there were significant increases in the mRNA expression of *cps*, presumably *cps* III (please see Chapter 1 and 8.4.2), in fish after 12 days of aestivation in air (~100-fold) or in mud (~160-fold) as compared with the freshwater control, but the mRNA expression of *cps* returned to control level by day 46 of aestivation in air or in mud (Fig. 8.1a). A similar pattern of changes in mRNA expression was observed for *ass* in the liver of fish aestivating in air or in mud (Fig. 8.1b). There were no significant differences in mRNA expressions of *cps* and *ass* between fish aestivating in air and fish aestivating in mud (Fig. 8.1a, b).

As for *gs*, a significant increase (~5-fold) in its mRNA expression occurred in fish after 12 days of aestivation in mud, but returned to control level by day 46 (Fig. 8.2a). Twelve or 46 days of aestivation in mud had no significant effects on the mRNA expression of *gs*. The mRNA expression of *gdh* was unaffected by 12 or 46 days of aestivation in air or in mud, but after 12 day of aestivation, the fold-change in *gdh* mRNA expression of fish aestivating in mud (1.7-fold) was significantly greater than that of fish aestivating in air (~0.85-fold) (Fig. 8.2b).

8.3.2. mRNA expression of cps *III,* ass*,* gs *and* gdh *in the liver of fish undergoing induction (3 or 6 days) and early maintenance (12 days) phases of aestivation in normoxia versus in hypoxia*

There were no significant differences in the Ct values and fold-changes in mRNA expression of the reference gene, *actin*, in the liver of *P. annectens* kept in freshwater (control), fish after 3, 6 or 12 days of aestivation in normoxia (in air) and fish after 3, 6 or 12 days of aestivation in hypoxia (2% O_2 in N_2) (Table 8.3).

For fish aestivating in normoxia, the fold-change in mRNA expression of *cps* was greater on day 3 (~270-fold) than on day 6 (~150-fold) and day 12 (~100-fold) (Fig. 8.3a). By contrast, the fold-change in mRNA expression of *cps* in fish after 12 days of aestivation in hypoxia remained high (~370-fold), and was significantly greater than that in fish after 12 days of aestivation in normoxia (Fig. 8.3a). In general, there were increases in mRNA expression of *ass* in fish during the 12 days of aestivation in normoxia or hypoxia (Fig. 8.3b). However, the fold-changes in mRNA expression of *ass* in fish aestivating in hypoxia were significantly greater than those in fish aestivating in normoxia on day 3 (21-fold versus 7-fold) and day 12 (23-fold versus 9-fold) (Fig.8.3b).

After 12 days of aestivation in hypoxia there was a significant increase in the mRNA-expression of *gs* in the liver of fish aestivating in hypoxia (13-fold) as compared with the freshwater control (Fig. 8.4a). Consequently, the fold-change in the mRNA-expression of *gs* in the liver of fish aestivating in hypoxia (13-fold) was significantly greater than that of fish aestivating in normoxia (2-fold) on day 12 (Fig. 8.4a). There were no significant changes in mRNA expression of *gdh* in the liver of fish during 12 days of aestivation in normoxia or hypoxia (Fig. 8.4b), although the fold-change in *gdh* mRNA expression in hypoxia was significantly greater than that in normoxia on day 12 (Fig. 8.4b).

8.3.3. mRNA expression of cps *III,* ass*,* gs *and* gdh *in the liver of fish undergoing the induction, maintenance and recovery phases of aestivation in air (normoxia)*

Overall, there were no significant differences in the Ct values and fold-changes in mRNA expression of the reference gene, *actin*, in the liver of *P. annectens* kept in freshwater

(control) and fish during the induction (3 or 6 days), early and prolonged maintenance (12 days and 6 months, respectively) and arousal (1, 3 or 6 days of recovery without food supply in water after 6 months of aestivation) phases of aestivation, despite consistent lower expression of this gene during the arousal phase (Table 8.4). Since this series of experiment involved tissue sampling from 3 different phases of aestivation spanning a period of ~6 months, it would be essential to examine the overall trend of changes instead of the exact change for each time point.

The general trend observed for the mRNA expression of *cps* in the liver of *P. annectens* was that increases occurred during the induction phase, but not during the prolonged maintenance phase (6 months) and arousal phase, of aestivation in air (Fig. 8.5a). As for *ass*, the observed trend was that there were increases in its mRNA expression which last through the prolonged maintenance (6 months) and arousal (1-6 days of recovery in water after 6 months of aestivation) phases (Fig. 8.5b).

The fold-changes in *gs* mRNA expression in the liver of *P. annectens* were relatively minor during the induction and maintenance phases of aestivation as compared with those of fish after 1-6 days of arousal from 6 months of aestivation (Fig. 8.6a). A similar trend was observed for the mRNA expression of *gdh*, except that the increase in expression began during the prolonged maintenance phase (6 months) of aestivation in air (Fig. 8.6b).

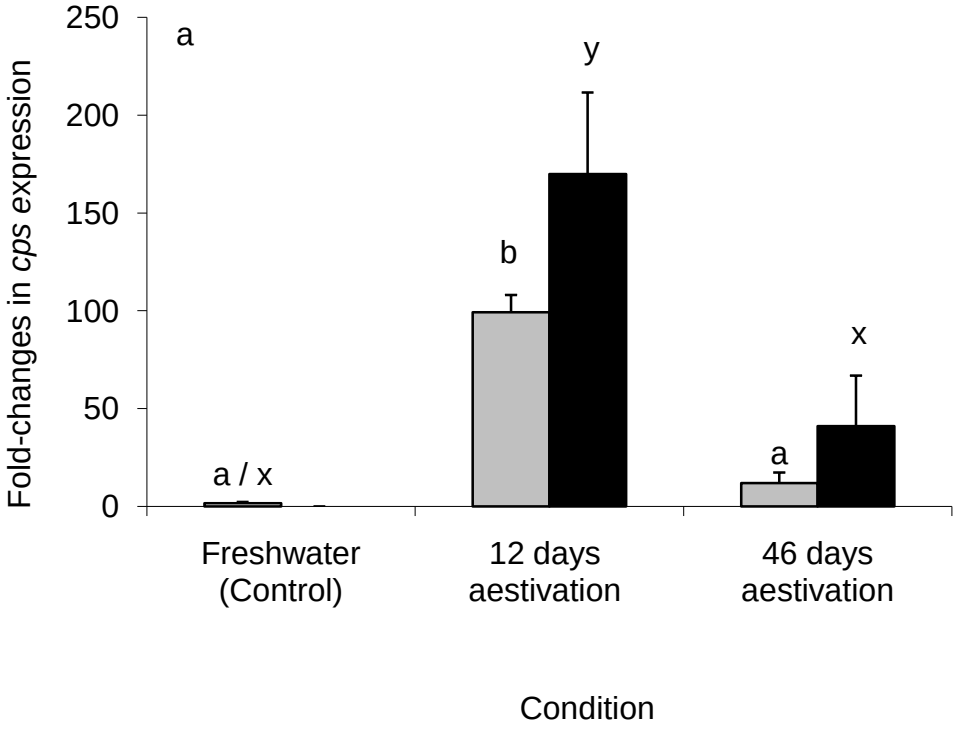
Table 8.2. Threshold cycle (C_T) and fold change in gene expression (calculated based on $2^{-\Delta C'_T}$) of *actin* in the liver of *Protopterus annectens* kept in freshwater (control), aestivated in air, or aestivated in mud for 12 or 46 days, inclusive of approximately 6 days of induction of aestivation.

	Freshwater (Control)	12 days aestivation		46 days aestivation	
		Air	Mud	Air	Mud
Threshold cycle (C_T) for actin	24.4 ± 0.3	24.2 ± 0.6	25.5 ± 0.2	25.2 ± 0.4	25.1 ± 0.7
Fold change in actin expression	1.1 ± 0.2	1.3 ± 0.4	0.46 ± 0.06	0.64 ± 0.22	0.91 ± 0.47

Results represent mean ± S.E.M. (*N*=4)

Fig. 8.1. Fold-changes in mRNA expression of (a) carbamoyl phosphate synthetase (*cps*) and (b) argininosuccinate synthetase (*ass*) in the liver of *Protopterus annectens* kept in freshwater (control) (white bars), aestivated in air (gray bars), or aestivated in mud (dark bars) for 12 or 46 days (both inclusive of approximately 6 days of induction phase of aestivation). Results represent mean + S.E.M. (N=4). Means not sharing the same letter (a and b) are significantly among control fish and fish aestivating in air (p<0.05). Means not sharing the same letter (x and y) are significantly different among control fish and fish aestivating in mud (p<0.05). * Significantly different from the corresponding value of fish aestivating in air (p< 0.05).

Fig. 8.1.


250
200
150
100
50
0
Fold-changes in cps expression
a
y
b
x
a
a / x
Freshwater (Control)
12 days aestivation
46 days aestivation
Condition

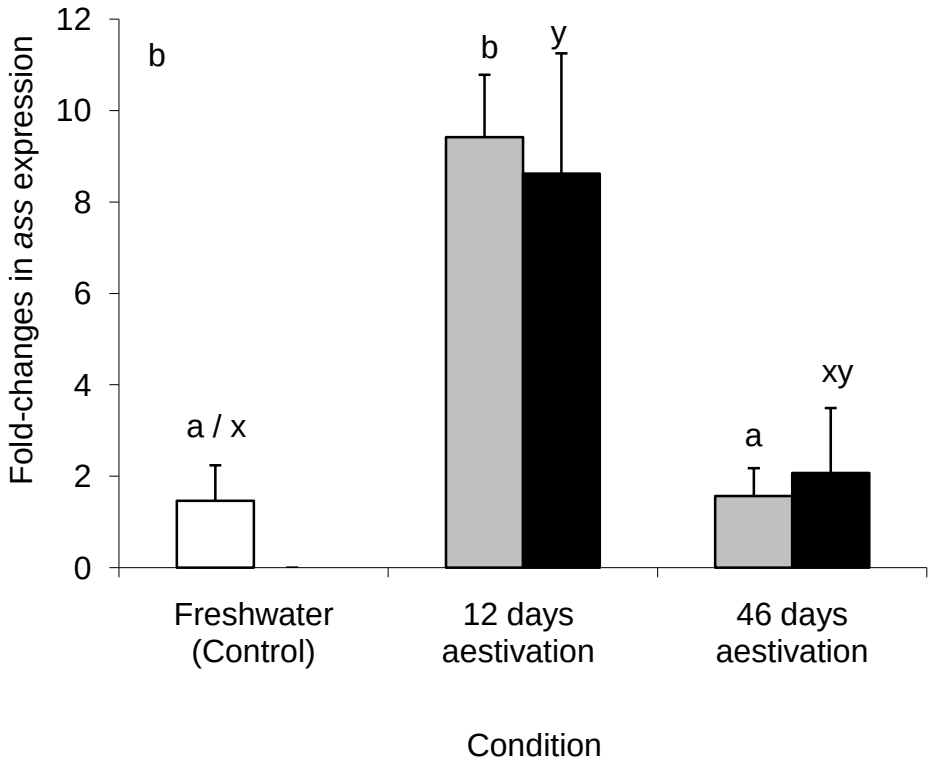
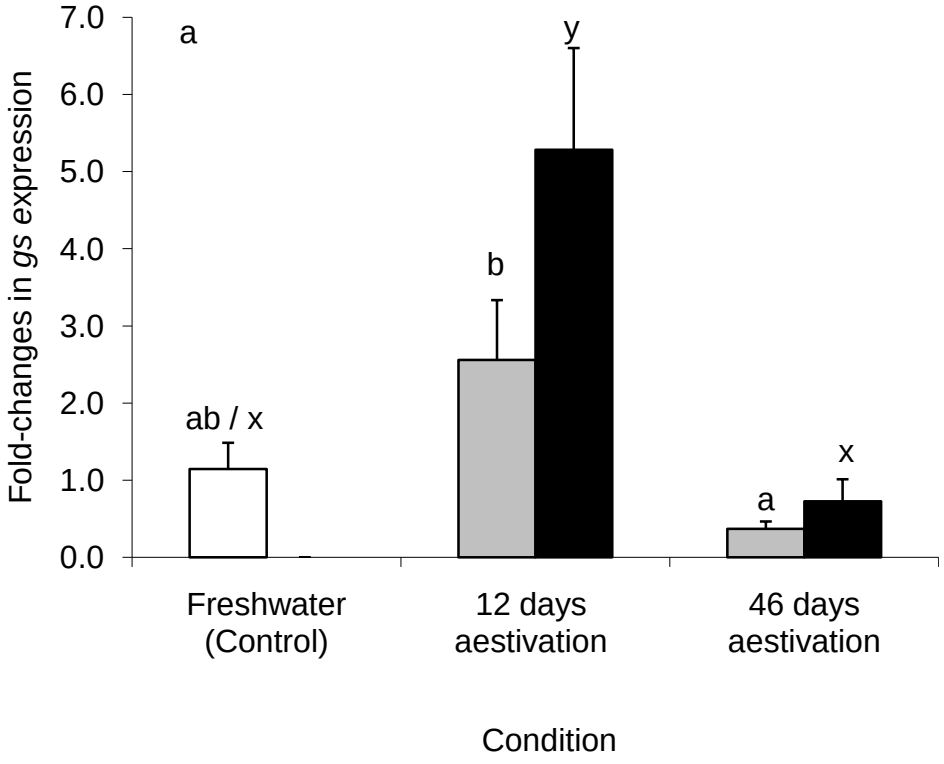

12
10
8
6
4
2
0
Fold-changes in ass expression
b
b
y
xy
a
a / x
Freshwater (Control)
12 days aestivation
46 days aestivation
Condition

Fig. 8.2. Fold-changes in mRNA expression of (a) glutamine synthetase (*gs*) and (b) glutamate dehydrogenase (*gdh*) in the liver of *Protopterus annectens* kept in freshwater (control) (white bars), aestivated in air (gray bars), or aestivated in mud (dark bars) for 12 or 46 days (both inclusive of approximately 6 days of induction phase of aestivation). Results represent mean + S.E.M. (N=4). Means not sharing the same letter (a and b) are significantly among control fish and fish aestivating in air (p<0.05). Means not sharing the same letter (x and y) are significantly different among control fish and fish aestivating in mud (p<0.05). * Significantly different from the corresponding value of fish aestivating in air (p< 0.05).

Fig. 8.2.


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fold-changes in gs expression
a
y
b
ab / x
a
x
Freshwater (Control)
12 days aestivation
46 days aestivation
Condition

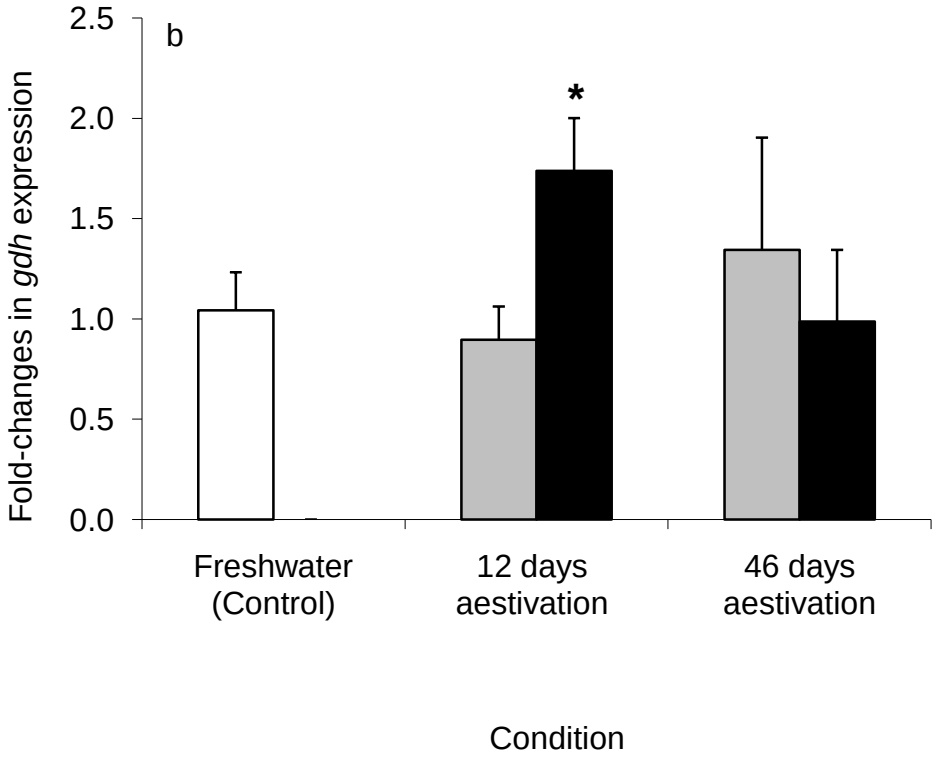

2.5
2.0
1.5
1.0
0.5
0.0
Fold-changes in gdh expression
b
*
Freshwater (Control)
12 days aestivation
46 days aestivation
Condition

Table 8.3. Threshold cycle (C_T) and fold change in gene expression (calculated based on $2^{-\Delta C'_T}$) of *actin* in the liver of *Protopterus annectens* during 12 days of induction (day 3 and day 6) and maintenance of aestivation (day 12) in normoxia or hypoxia (2% O_2 in N_2) as compared with control fish kept in freshwater.

	Freshwater (Control)	Normoxia			Hypoxia		
		Day 3	Day 6	Day 12	Day 3	Day 6	Day 12
Threshold cycle (C_T) for actin	24.4 ± 0.3	24.2 ± 0.4	24.2 ± 0.5	24.3 ± 0.6	24.6 ± 0.2	24.5 ± 0.5	23.8 ± 0.2
Fold change in actin expression	1.1 ± 0.2	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	0.9 ± 0.1	1.0 ± 0.2	1.5 ± 0.2

Results represent mean ± S.E.M. (*N*=4)

Fig. 8.3. Fold-changes in mRNA expression (a) carbamoyl phosphate synthetase (*cps*) and (b) argininosuccinate synthetase (*ass*) in the liver of *Protopterus annectens* during 12 days of aestivation, inclusive of the induction phase (day 3 and day 6) and maintenance phase (day 12), in normoxia (gray bars) or hypoxia (2% O_2 in N_2) (dark bars) as compared with control fish kept in freshwater (white bars). Results represent mean + S.E.M. (*N*=3 for *cps*; *N*=4 for *ass*). Means not sharing the same letter (a, b and c) are significantly different among control fish and fish aestivating in normoxia ($p<0.05$). Means not sharing the same letter (x and y) are significantly different among control fish and fish aestivating in hypoxia ($p<0.05$). *Significantly different from the corresponding value of fish aestivating in normoxia ($p< 0.05$).

Fig. 8.3.

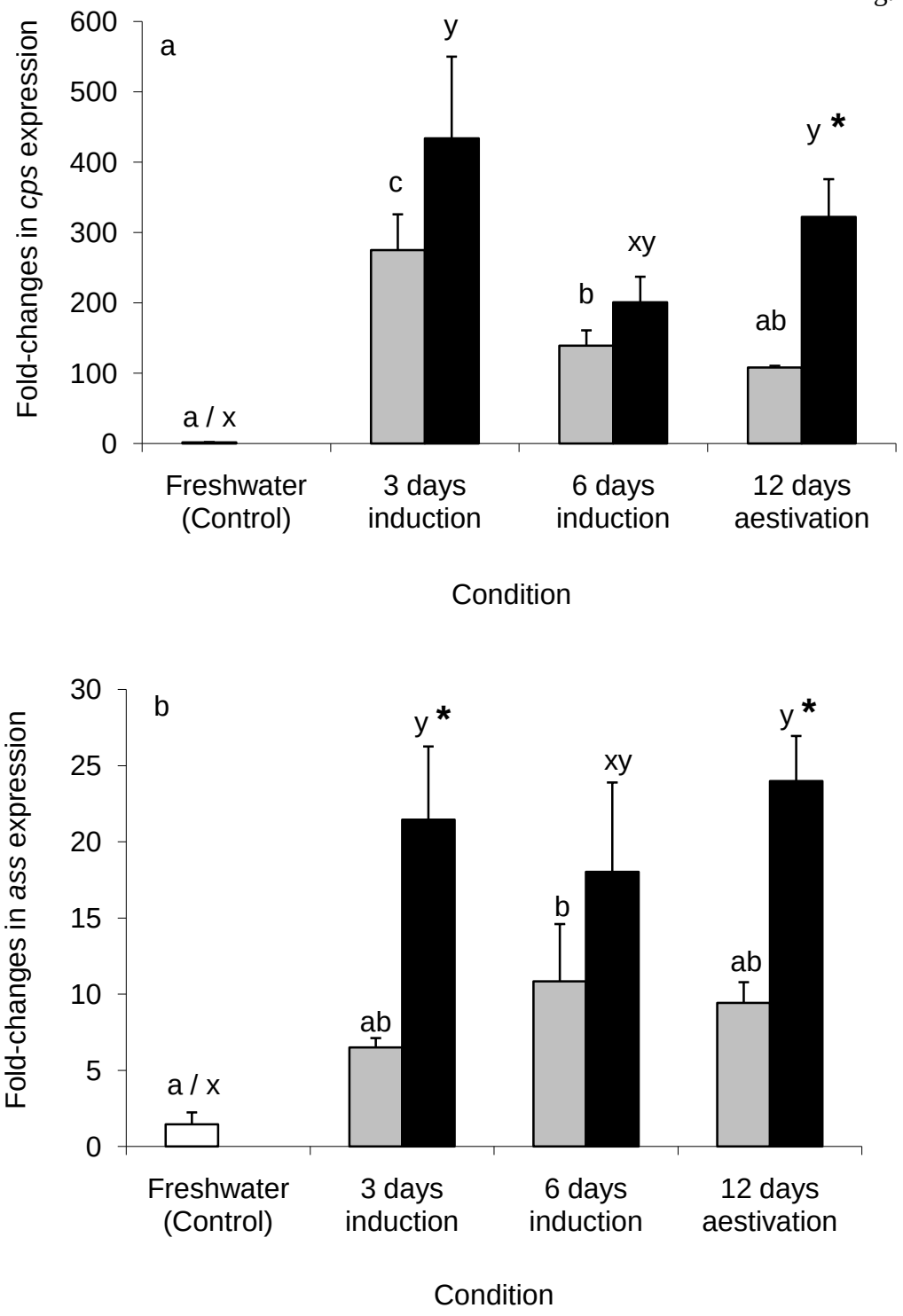
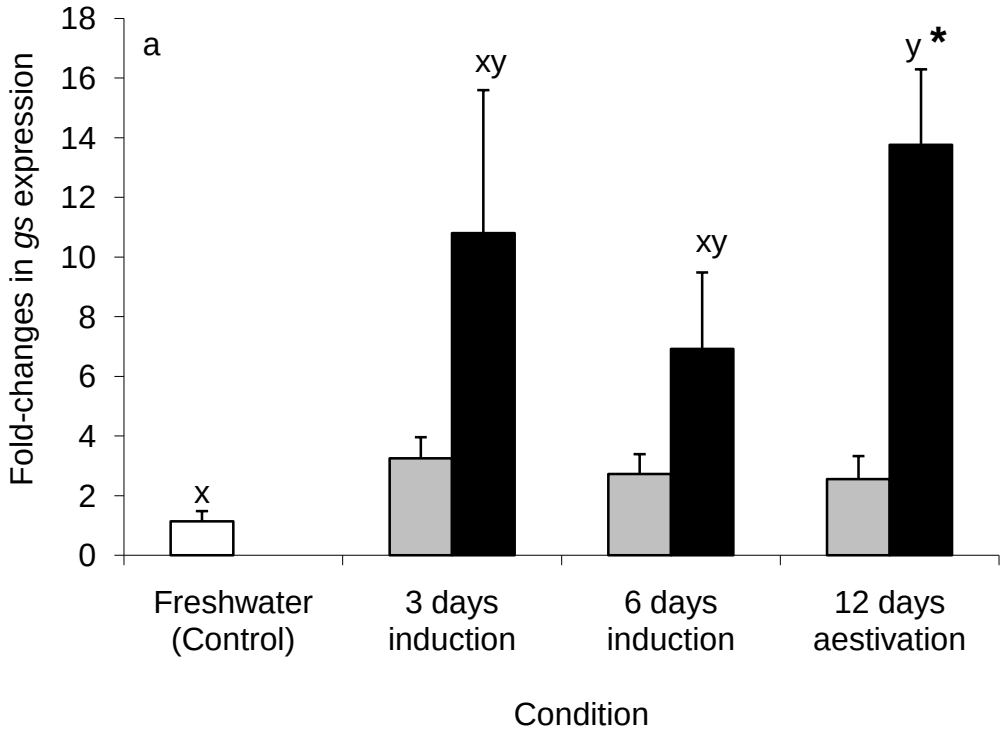

Fold-changes in cps expression
600
500
400
300
200
100
0
a
a / x
y
c
xy
b
y *
ab
Freshwater (Control)
3 days induction
6 days induction
12 days aestivation
Condition
Fold-changes in ass expression
30
25
20
15
10
5
0
b
a / x
y *
ab
xy
b
y *
ab
Freshwater (Control)
3 days induction
6 days induction
12 days aestivation
Condition

Fig. 8.4. Fold-changes in mRNA expression (a) glutamine synthetase (*gs*) and (b) glutamate dehydrogenase (*gdh*) in the liver of *Protopterus annectens* during 12 days of aestivation, inclusive of the induction phase (day 3 and day 6) and maintenance phase (day 12), in normoxia (gray bars) or hypoxia (2% O_2 in N_2) (dark bars) as compared with control fish kept in freshwater (white bars). Results represent mean + S.E.M. (N=4). Means not sharing the same letter (a, b and c) are significantly different among control fish and fish aestivating in normoxia (p<0.05). Means not sharing the same letter (x and y) are significantly different among control fish and fish aestivating in hypoxia (p<0.05). *Significantly different from the corresponding value of fish aestivating in normoxia (p< 0.05).

Fig. 8.4.


a
Fold-changes in gs expression
18
16
14
12
10
8
6
4
2
0
x
xy
xy
y *
Freshwater (Control)
3 days induction
6 days induction
12 days aestivation
Condition

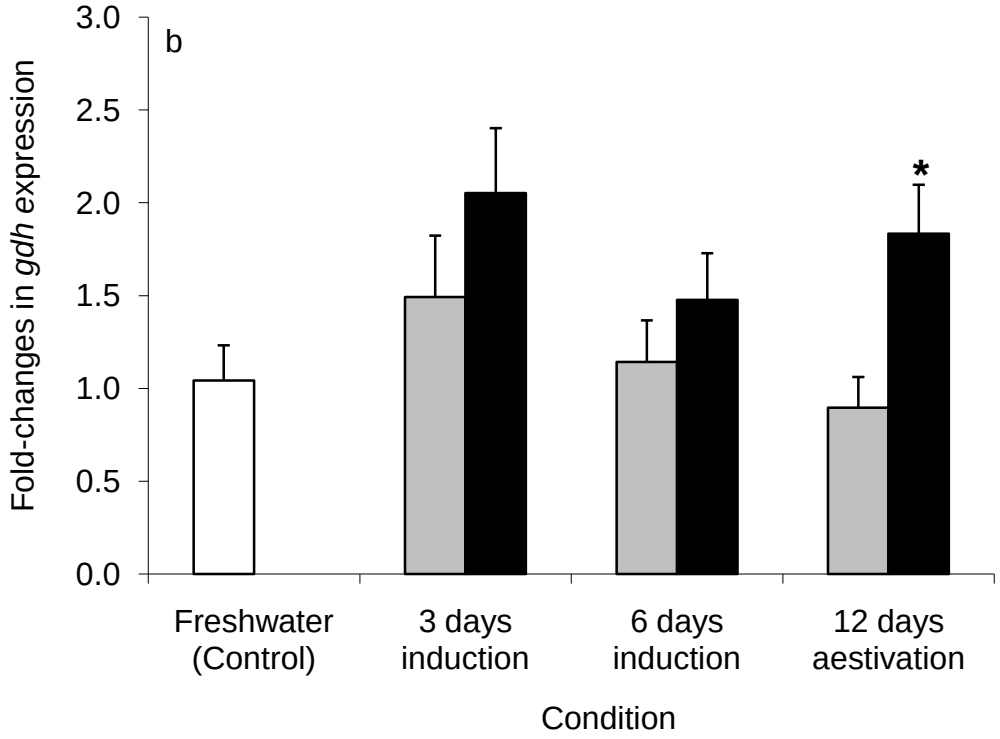

b
Fold-changes in gdh expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Freshwater (Control)
3 days induction
6 days induction
12 days aestivation
Condition

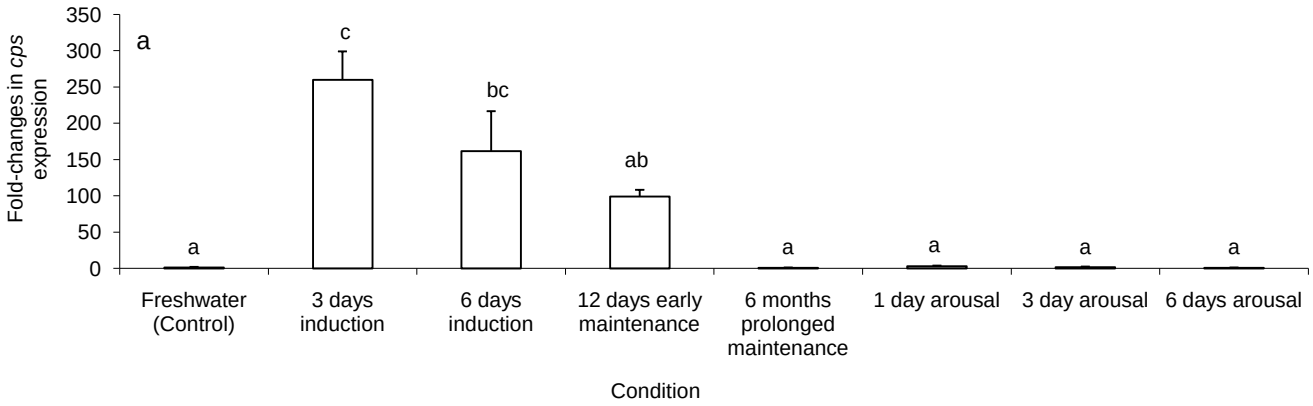
Table 8.4. Threshold cycle (C_T) and fold change in gene expression (calculated based on $2^{-\Delta C'_T}$) of *actin* in the liver of *Protopterus annectens* kept in freshwater (control; day 0), or after 3 or 6 days of induction phase of aestivation, or after 12 days (early maintenance) or 6 months (prolonged maintenance) of maintenance phase of aestivation, or after 1 day, 3 days or 6 days of arousal phase of aestivation (in freshwater without food after arousal from 6 months of aestivation).

	Freshwater (Control)	Induction phase		Maintenance phase		Arousal phase		
		3 days	6 days	12 days	6 months	1 day	3 days	6 days
Threshold cycle (C_T) for actin	24.4 ± 0.3	24.2 ± 0.4	24.2 ± 0.6	24.3 ± 0.6	23.8 ± 0.1	25.4 ± 0.5	25.9 ± 0.1	25.6 ± 0.4
Fold change in actin expression	1.1 ± 0.2	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.5 ± 0.1	0.58 ± 0.21	0.35 ± 0.04	0.49 ± 0.13

Results represent mean ± S.E.M. (*N*=4)

Fig. 8.5. Fold-changes in mRNA expression of (a) carbamoyl phosphate synthetase (*cps*), and (b) argininosuccinate synthetase (*ass*) in the liver of *Protopterus annectens* kept in freshwater (control; day 0), or after 12 days (early maintenance) or 6 months (prolonged maintenance) of maintenance phase of aestivation, or after 1 day, 3 days or 6 days of arousal phase of aestivation (in freshwater without food after arousal from 6 months of aestivation). Results represent mean + S.E.M. (N=4). Means not sharing the same letter are significantly different (p<0.05).

Fig. 8.5.


a
Fold-changes in cps expression
350
300
250
200
150
100
50
0
a
c
bc
ab
a
a
a
a
Freshwater (Control)
3 days induction
6 days induction
12 days early maintenance
6 months prolonged maintenance
1 day arousal
3 day arousal
6 days arousal
Condition

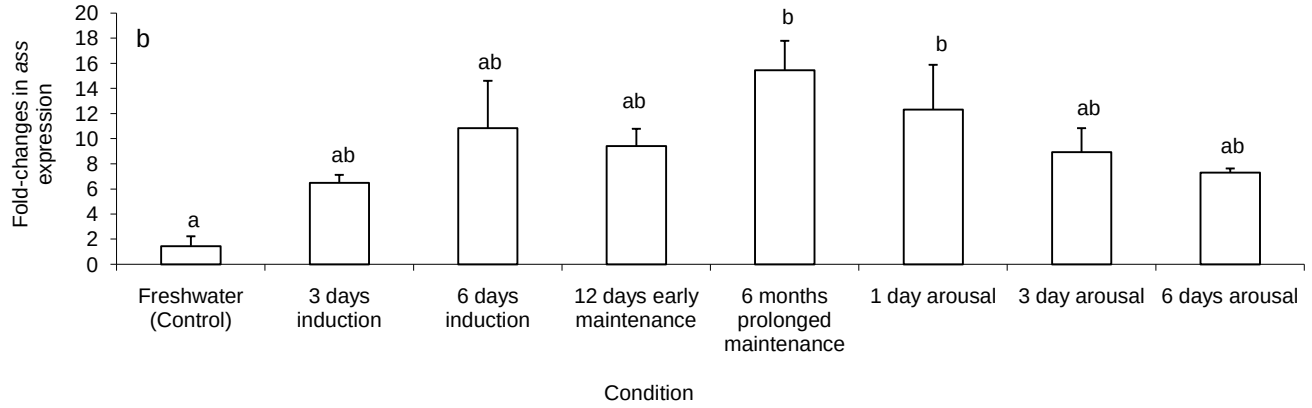
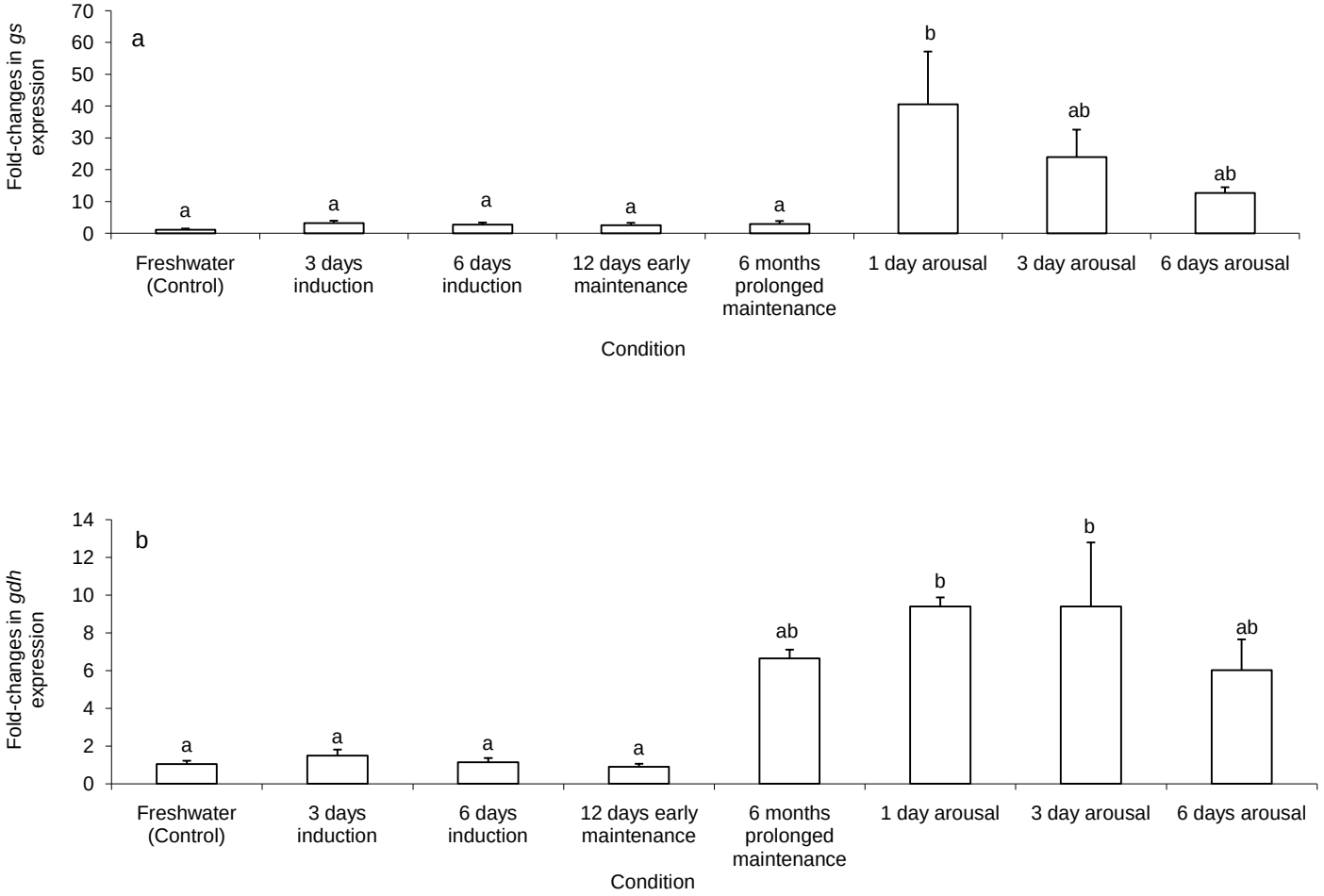

b
Fold-changes in ass expression
20
18
16
14
12
10
8
6
4
2
0
a
ab
ab
ab
b
b
ab
ab
Freshwater (Control)
3 days induction
6 days induction
12 days early maintenance
6 months prolonged maintenance
1 day arousal
3 day arousal
6 days arousal
Condition

Fig. 8.6. Fold-changes in mRNA expression of (a) glutamate synthetase (*gs*), and (b) glutamate dehydrogenase (*gdh*) in the liver of *Protopterus annectens* kept in freshwater (control; day 0), or after 3 or 6 days of induction phase of aestivation, or after 12 days (early maintenance) or 6 months (prolonged maintenance) of maintenance phase of aestivation, or after 1 day, 3 days or 6 days of arousal phase of aestivation (in freshwater without food after arousal from 6 months of aestivation). Results represent mean + S.E.M. (N=4). Means not sharing the same letter are significantly different (p<0.05).


a
Fold-changes in gs expression
70
60
50
40
30
20
10
0
a
a
a
a
a
b
ab
ab
Freshwater (Control)
3 days induction
6 days induction
12 days early maintenance
6 months prolonged maintenance
1 day arousal
3 day arousal
6 days arousal
Condition
b
Fold-changes in gdh expression
14
12
10
8
6
4
2
0
a
a
a
a
ab
b
b
ab
Freshwater (Control)
3 days induction
6 days induction
12 days early maintenance
6 months prolonged maintenance
1 day arousal
3 day arousal
6 days arousal
Condition

8.4. Discussion

8.4.1. mRNA expression of CPS and ASS and the capacity of OUC in the liver of P. annectens *during 12 or 46 days of aestivation in air versus in mud*

Results obtained in this study demonstrated that increases in the rate of urea synthesis and in the activities of CPS III and ASS (and argininosuccinate lyase) in the liver of aestivating *P. annectens* (Long et al., 2005; 2008a, b), and probably also other African lungfishes (Chew et al., 2004, Ip et al., 2004), involved up-regulation of mRNA expression of some crucial OUC enzymes. This implies that the OUC capacity was up-regulated since there could be increases in expression of related enzymes at the transcriptional and perhaps also the translational levels.

Judging by the fold-changes in mRNA expression, CPS was more critically controlled than ASS in the OUC of *P. annectens*. For fish aestivating in air and mud, the fold changes of *cps* (100- to 160-fold) was extraordinarily high, and it was much greater than that of *ass* (8- to 9-fold) on day 12. This would indicate that CPS could have an important regulatory role in increased urea synthesis. This proposition is subsequently confirmed by the observation that the mRNA expression of *cps*, but not *ass*, return to control level during the prolonged maintenance phase of aestivation in air (8.4.5.). The increase in mRNA expression in the liver of aestivating *P. annectens* probably represents the highest fold-change known among ureogenic animals exposed to stressful conditions that could induce OUC capacity.

There is an apparent controversy between the magnitudes of increases in *cps* and *ass* mRNA expression and the magnitudes of increases in CPS and ASS activities (Chapter 2; Loong et al., 2008b), the former being much greater than the latter. One of the possible explanations is that the increase in transcription was not accompanied with a similar magnitude of increase in translation of the *cps* and *ass* genes. Indeed, judging from the SSH results (Chapter 4), there could be a decrease in translational activities, in general, in the liver

of *P. annectens* during the induction phase of aestivation (day 6), and such a change in translational activities could be an essential adaptation of the aestivation process.

On day 46, the mRNA expression of *cps* and *ass* returned to control levels in the liver of fish that had undergone the early maintenance phase of aestivation. These results are in support of previous conclusions that increased urea synthesis occurs at the greatest rate during the induction phase, and that decreased ammonia production is more crucial during the maintenance phase, of aestivation (Chapters 1, 2 and 3; Loong et al. 2005, 2008a, b; Chew et al., 2004; Ip et al., 2005f). They also provide an explanation to why Janssens and Cohen (1968a, b) reported that there was no change in the rate of urea synthesis in *P. aethiopicus* during the maintenance phase of aestivation as compared with the control. Since a complete cocoon is formed on day 6, results obtained from this study on day 12 represent a combined effect of 6 days of induction plus 6 days of aestivation. In order to examine this phenomenon in greater detail, it was therefore important to extend the experiment to day 3 and day 6 (see 8.4.3 below).

Presumably, fish aestivating in mud would encounter hypoxia, resulting in low efficiencies of energy metabolism (Chapter 2; Loong et al., 2008b). Since urea synthesis through the OUC is energy-intensive, i.e. 5 mol of ATP is required for each mole of urea synthesized through CPS III involving GS, it would be disadvantageous for *P. annectens* aestivating in mud to maintain an OUC capacity similar to the OUC capacity of fish aestivating in air, if the emphasis was solely on energy conservation. Indeed, it has been demonstrated previously that the non-ureogenic swamp eel, *Monopterus albus*, which is capable of detoxifying ammonia to glutamine in and outside the brain, accumulates much less glutamine, the formation of which is also ATP-dependent, in its tissues during aestivation in mud as compared to during aerial exposure (Chew et al., 2004). *M. albus* depends more on a reduction in endogenous ammonia production than an increase in glutamine formation to avoid ammonia toxicity during aestivation in mud (Chew et al., 2004). Theoretically, *P.*

annectens could adopt a strategy similar to that of *M. albus*, by suppressing the mRNA expression of *cps* and *ass* to reduce the rate of urea synthesis. However, since that was not the case, it is logical to conclude that increased urea synthesis and accumulation is a process intrinsic and essential to aestivation, which cannot be compromised even during aestivation in mud (or in hypoxia). Hence, it is logical to conclude that urea has some kind of physiological function besides being a nitrogenous waste in aestivating *P. annectens*. These results corroborate the proposition of Ip et al. (2005d) that urea accumulated during the induction and early maintenance phases of aestivation in African lungfish could be one of the important endogenous signals that initiate the aestivation process.

8.4.2. Pattern of change in mRNA expression of gs *in the liver of* P. annectens *during 12 or 46 days of aestivation in air or in mud and its implication*

Traditionally, the classification of CPS is predominantly dependent on whether it utilizes NH_4^+ (CPS I) or glutamine (CPS III) as a substrate, is activated by N-acetylglutamate, and is refractory to UTP inhibition. Based on these criteria, Loong et al. (2005; Chapter 1) concluded that the CPS present in the liver of *P. annectens* was actually CPS III, which is contrary to the report by Mommsen and Walsh (1989) on the presence of CPS I in the liver of this lungfish. In this study, the increase in mRNA expression of *cps* in the liver occurred in association with increase in mRNA expression of *gs*, especially in fish after 12 days of aestivation in mud. Since there was a lack of glutamine accumulation in the liver of these fish (Chapter 2), it can be concluded that increased glutamine synthesis, if it occurred, was channeled into CPS III of the OUC for increased urea synthesis. Hence, these results are in support of the conclusion that *P. annectens* possesses CPS III (Loong et al., 2005), instead of CPS I (Mommsen and Walsh, 1989). Furthermore, the up-regulation of *gs* mRNA expression in *P. annectens* after 12 days of aestivation in air or in mud further support the proposition

that the adaptations during aestivation in mud exhibited by *P. annectens* differ from those exhibited by *M. albus*.

8.4.3. mRNA expression of cps, ass *and* gs *in the liver of* P. annectens *during the induction and early maintenance phases of aestivation in normoxia versus in hypoxia*

For fish that underwent aestivation in normoxia (in air), the increase in *cps* mRNA expression peaked (~270-fold) on day 3 (the induction phase) and returned to control level by day 12. As for *ass*, the increase in mRNA expression peaked on day 6 instead. Taken together, it can be confirmed that there are differences in regulation of mRNA expression between *cps* and *ass*. In addition, these results confirm that increased urea synthesis and up-regulation of OUC capacity occurred mainly during the induction phase of aestivation in normoxia

More importantly, results from this study confirm that aestivation in normoxia and aestivation in hypoxia had different effects on *P. annectens*. The increases of mRNA expression for *cps* and *ass* were sustained from the induction phase (day 3) into the early maintenance phase (day 12) of aestivation in hypoxia. Consequently, there were significantly greater fold-changes of *cps* and *ass* mRNA expression in fish undergoing 12 days of aestivation in hypoxia as compared with fish undergoing 12 days of aestivation in normoxia. Surprisingly, a phenomenal level of ~370-fold in change in mRNA expression of *cps* was registered on day 3 and day 12 of aestivation in hypoxia. These results have several implications. Firstly, it can be confirmed that increased urea synthesis and up-regulation of OUC capacity were essential to aestivation, as they were not compromised during exposure to severe hypoxia (2% O_2 in N_2). Secondly, considering the proposition that increased synthesis and accumulation of urea is crucial to the induction of aestivation (Ip et al., 2005f), it can also be deduced that induction of aestivation in fish aestivating in hypoxia would be more effective than that in fish aestivating in normoxia. Thirdly, the greater increase in

mRNA expression of *cps* and *ass* in fish aestivating in hypoxia compared with fish aestivating in normoxia could indicate that the former could have a greater reduction in metabolic rate than the latter. It has been well established that hypoxia could be an effective signal to induce a reduction in metabolic rate in animals, and reduction in metabolic rate could involve a suppression of translational processes and protein synthesis (Hochachka, 1990). Hence it would be essential for fish aestivating in hypoxia to up-regulate mRNA expression of *cps* and *ass* to higher levels in order to increase the level of protein expression comparable to or higher than those in fish aestivating in normoxia.

The >10-fold up-regulation of *gs* mRNA expression in fish aestivating in hypoxia once again corroborate the proposition that CPS III was involved in the hepatic OUC of *P. annectens*. Furthermore, it supports the conclusion that the strategy adopted by *P. annectens* differs from that adopted by *M. albus* (see section 8.4.1.).

8.4.4. The lack of changes in mRNA expression of **gdh** *during the induction and early maintenance phase of aestivation and its implication*

Results from this study indicate that the mRNA expression of *gdh* in the liver of *P. annectens* was unaffected by 46 days of aestivation in air or in mud, or by 12 days of aestivation in normoxia or hypoxia. The significant differences in *gdh* mRNA expression on day 12 of aestivation in air or in mud, and on day 12 of aestivation in normoxia or hypoxia were probably too small (~1.8-fold) to be of physiological significance. This would imply that the regulation of hepatic *gdh* during the induction and early maintenance phase of aestivation did not involve an up-regulation of gene expression, and occurred most likely by post-transcriptional and post-translational modification (see Chapter 3).

8.4.5. mRNA expression of **cps, ass, gs** *and* **gdh** *in the liver of P. annectens during the induction, maintenance and arousal phases of aestivation in air*

Results of this set of experiments were the most revealing. It become immediately apparent that CPS, and not ASS, could be the regulatory enzyme of the hepatic OUC in *P. annectens*, since the mRNA expression of *cps* returned to the control level after 6 months of aestivation in air or during 6 days of arousal from 6 months of aestivation. The explanation for the similar rates of urea synthesis between fish undertaking prolonged aestivation and control fish (Janssens and Cohen, 1968a, b) can be justified by the mRNA expression of *cps* but not by the mRNA expression of *ass*.

It is important to note that there was a prominent increase in mRNA expression of *gs* in the liver of fish during the first day of arousal from 6 months of aestivation in air. Unlike results obtained from the other 2 sets of experiments in this study, the increase in mRNA expression of *gs* did not complement that of an increase in mRNA expression of *cps*. It can be concluded from these results that there could be an increase in the synthesis of glutamine upon arousal and that the excess glutamine synthesized was not channeled into the OUC during the arousal phase as in the case of the induction phase. Upon arousal, it is important for the fish to undergo repair and regeneration of its tissues that could have been modified and/or damaged during the induction and prolonged maintenance phases of aestivation. Glutamine has many functions and is involved in the synthesis of many biochemicals, including purines and pyrimidines which could be important for cell proliferation and tissue regeneration. Hence, it is predictable that the increase in GS activity, if it indeed occurred, would not lead to accumulation of glutamine in tissues during the arousal phase of aestivation. Instead, it would be channeled into the biosynthesis of other important compounds.

A keen observation of fish aroused from aestivation revealed that they would not feed until 7-10 days later. This would mean that cell proliferation and tissue regeneration could only be sustained by endogenous resources and protein is logically an important source of energy supply and amino acids for protein synthesis. During the arousal phase, it would be

essential to conserve nitrogen (N) for anabolic purposes and hence there could be an increase in rate of nitrogen metabolism, and thus amino acid metabolism, without an increase in the rate of N-waste production. Hepatic GDH is the most crucial enzyme involved in amino acid metabolism and is involved in transdeamination of many amino acids. Indeed, there was a great increase in *gdh* mRNA expression in the liver of *P. annectens* during the first 3 days of arousal from 6 months of aestivation in air. More importantly, the overall trend of mRNA expression suggests that the increase in *gdh* mRNA expression would have been initiated during the prolonged maintenance phase of aestivation (6 months). Hence, it could be deduced that either these fish had to mobilize amino acids through increased GDH activity to sustain prolonged aestivation or they increased GDH activity in anticipation of arousal after a prolonged period of aestivation, the confirmation of which awaits future studies.

8.5. Summary

This study aimed to provide definitive evidence to support the proposition that physiological phenomena involved in aestivation, e.g. increased urea synthesis, could vary between different phases of aestivation and differ between normoxic and hypoxic conditions. Results obtained indicate that mRNA expression of *cps*, *ass* as OUC enzymes and *gs*, the enzyme that supplies glutamine to CPS III, were up-regulated during the induction phase and earlier maintenance phase (3-12 days) of aestivation in air, in mud and in hypoxia. Aestivation in normoxia and aestivation in hypoxia had different effects on *P. annectens*. The increases of mRNA expression for *cps* and *ass* were sustained from the induction phase (day 3) into the early maintenance phase (day 12) of aestivation in hypoxia. Consequently, there were significantly greater fold-changes of *cps* and *ass* mRNA expression in fish undergoing 12 days of aestivation in hypoxia as compared with fish undergoing 12 days of aestivation in normoxia. On the other hand, the mRNA expression of *gdh* in the liver of *P. annectens* was unaffected by 46 days of aestivation in air or in mud, or by 12 days of aestivation in normoxia or hypoxia. This would imply that the regulation of hepatic *gdh* during the induction and early maintenance phase of aestivation did not involve an up-regulation of gene expression, and occurred most likely by post-transcriptional and post-translational modification. During the first day of arousal from 6 months of aestivation in air, there was a prominent increase in mRNA expression of *gs* in the liver of fish. Since increase in mRNA expression of *gs* did not compliment that of an increase in mRNA expression of *cps*, it is probable that the excess glutamine synthesized was not channeled into the OUC during the arousal phase as in the case of the induction phase. Increased glutamine synthesis could be important for cell proliferation and tissue regeneration, as it is involved in the synthesis of many biochemicals, including purines and pyrimidines. In addition, there was a great increase in *gdh* mRNA expression in the liver of *P. annectens* during the first 3 days of arousal from 6 months of aestivation in air. The overall trend of mRNA expression suggests

that the increase in *gdh* mRNA expression would have been initiated during the prolonged maintenance phase of aestivation (6 months). Hence, it could be deduced that either these fish had to mobilize amino acids through increased GDH activity to sustain prolonged aestivation or they increased GDH activity in anticipation of arousal after a prolonged period of aestivation.

9. Chapter 6:

Overall integration, synthesis and conclusions

9.1. Nitrogen metabolism and excretion during the induction phase

9.1.1. Urea as an internal signal in the induction process

Results presented in Chapters 1-5 indicate that increased urea synthesis was an important process of aestivation in *P. annectens* even during aestivation in mud or in hypoxia with inefficient energy metabolism. However, based on the mRNA expression of *cps* (Chapters 4 and 5), it can be concluded that increased urea synthesis was particularly important to the induction phase (and perhaps the early maintenance phase) of aestivation but not so to the prolonged maintenance and arousal phases.

Although aestivation normally occurs in association with summer heat, it is not part of a chronobiological rhythm but an episodic event that requires an initial stimulus. To understand the process of aestivation, it is important to distinguish the induction phase from the maintenance phase, but neither inducing factors nor maintaining mechanisms are fully understood for aestivating animals (Fishman et al., 1987). Several inducing factors of aestivation have been proposed for African lungfishes (Fishman et al., 1987), which include (1) dehydration, leading to oliguria/anuria and metabolic acidosis, (2) air-breathing on land, leading to CO_2 retention and respiratory acidosis, (3) starvation, affecting the metabolic, circulatory and respiratory changes and (4) stress, leading to release of neurohormonal mediators and/or affecting thyroid function. Recent works reveal that increases in environmental ammonia concentration (Chew et al., 2005b; Ip et al., 2005d) and ambient salinity (Ip et al., 2005c) could be important environmental cues for the induction of aestivation in African lungfish.

Naturally, aestivation occurs when an African lungfish is stranded in a puddle of water or in semi-solid mud during the dry season. The continual excretion of ammonia into a small volume of external medium would lead to high concentrations of environmental ammonia. The situation would be aggravated by the constant evaporation of the external medium under high temperature, further concentrating ammonia and other ions and resulting

in high ambient salinity. Indeed, Ip et al. (2005c) demonstrated that *P. dolloi* exposed to 3‰ water for 6 days exhibited consistently lower daily urea excretion rate as compared with the freshwater control. Simultaneously, there were decreases in urea contents in various tissues and organs. Ip et al. (2005c) therefore concluded that *P. dolloi* could respond to salinity changes in the external medium as it dried up, suppressing ammonia production in preparation of aestivation. In a separate study, Chew et al. (2005b) discovered that *P. dolloi* was capable of maintaining low concentrations of ammonia in its body by up-regulating the rate of urea synthesis to detoxify ammonia when exposed to environmental ammonia. Simultaneously, *P. dolloi* was able to increase its rate of urea excretion, but urea accumulated in the muscle, liver, and plasma of specimens exposed to environmental ammonia despite the significant increase in urea excretion rate (Chew et al., 2005b). Similar observations were made on *P. dolloi* fasted for 40 days, and urea contents in various tissues increased significantly in fasted *P. dolloi* (Chew et al., 2004), despite being immersed in water and having the capacity to up-regulate urea excretion under certain conditions (Lim et al., 2004; Wood et al., 2005b). Since fasting is known to be one of the inducing factors of aestivation, urea accumulation could be an important part of the induction mechanism.

Ip et al. (2005d) undertook a series of experiments that aimed to determine whether ammonia (as NH_4Cl) injected intra-peritoneally into *P. dolloi*, would be excreted directly instead of being detoxified to urea, and to examine whether injected urea would be retained in this lungfish, leading to decreases in liver arginine and brain tryptophan levels as observed during aestivation on land. Despite being ureogenic, *P. dolloi* rapidly excreted the excess ammonia within the subsequent 12 h after NH_4Cl was injected into its peritoneal cavity. By contrast, when urea was injected intra-peritoneally into *P. dolloi*, only a small percentage (34%) of it was excreted during the subsequent 24-h period. At h 24, significant quantities of urea were retained in various tissues of *P. dolloi*. Injection with urea led to an apparent reduction in endogenous ammonia production, a significant decrease in the hepatic arginine

content, and a significantly lower level of brain tryptophan in this lungfish. All these three phenomena had been observed in aestivating *P. dolloi* (Chew et al., 2004). Therefore, Ip et al. (2005d) concluded that urea synthesis and accumulation could be one of the essential factors in initiating and maintaining aestivation.

Similarly, Hiong et al. (2005) reported that the giant African snail, *Achatina fulica*, accumulated urea progressively not only during 23 days of aestivation, but also during 23 days of fasting (Hiong et al., 2005). Fasting did not impede ammonia or urea excretion in *A. fulica* and fasting snails moved around in the containers actively with part of their bodies fully extended out of the shell. Yet, urea accumulation occurred in the hepatopancreas and foot muscle, with ammonia levels remained relatively unchanged. Hence, similar to African lungfishes (Chew et al., 2005b; Ip et al., 2005d), urea might be involved as part of the induction mechanism of aestivation, which is unrelated to nitrogenous excretion and water retention, in *A. fulica* (Hiong et al. 2005).

9.1.2. Changes in the permeability of the skin to ammonia and its implications

Results presented in Chapters 1 and 2 indicated that there could be a decrease in the rate of ammonia excretion during aerial exposure or the induction phase of aestivation. There could be several explanations for this phenomenon. Firstly, ammonia could be accumulating in the external medium during the induction phase of aestivation and the elevated ammonia concentration in the external medium impeded ammonia excretion. Secondly, endogenous ammonia was converted to urea leading to a decrease in ammonia excretion rate. Thirdly, decreased ammonia excretion implies decreased ammonia permeability in order to trap ammonia and turn it into urea. The third explanation is unorthodox and to my knowledge it has never been explored before.

Since African lungfishes would have to defend against environmental ammonia toxicity during the induction phase of aestivation, Loong et al. (2007) undertook a study to

determine how the African lungfish, *Protopterus aethiopicus*, defended against ammonia toxicity when confronted with high concentrations (30 or 100 mmol l^{-1}) of environmental ammonia. Using an Ussing-like apparatus, they (Loong et al., 2007) reported that the skin of *P. aethiopicus* had low permeability (1.26 x 10^{-4} μmol min^{-1} cm^{-1}) to NH_3 *in vitro*. Indeed, the influx of exogenous ammonia into fish exposed to 30 mmol l^{-1} NH_4Cl was low (0.117 μmol min^{-1} 100 g^{-1} fish). As a result, *P. aethiopicus* could afford to maintain relatively low ammonia contents in plasma, muscle, liver and brain even after 6 days of exposure to 100 mmol l^{-1} NH_4Cl. In addition, Loong et al. (2007) obtained results which suggest that *P. aethiopicus* was capable of decreasing the NH_3 permeability of its body surface in response to ammonia exposure. After 6 days of exposure to 100 mmol l^{-1} NH_4Cl, the NH_3 permeability constant of the skin (0.55 x 10^{-4} μmol min^{-1} cm^{-1}) decreased to half of that of the control. A decrease in the already low cutaneous NH_3 permeability and an increased urea synthesis, working in combination, allowed *P. aethiopicus* to effectively defend against environmental ammonia toxicity without elevating the plasma ammonia level. Hence, unlike other fishes (see Ip et al., 2001, 2004a, b; Chew et al., 2006 for reviews), glutamine and alanine contents did not increase in the muscle and liver, and there was no accumulation of glutamine in the brain, even when the fish was immersed in water containing 100 mmol l^{-1} NH_4Cl (Loong et al., 2007).

The mechanisms involved in the alteration of NH_3 permeability in the skin of *P. aethiopicus* are unclear at present, but results obtained by Loong et al. (2007) implies that the permeability of the skin to water and ions can be altered during the induction and perhaps also the arousal phases of aestivation. Similar to the low NH_3 permeability in the skin of *P. aethiopicus*, the body surface of *P. dolloi* has low permeability to water and ions (Wilkie, 2007). Recently, Wilkie et al. (2007) and Staples et al. (2008) studied *P. dolloi* in water (control) or exposed to air for an extended period (5 months) without the formation of a complete cocoon. In essence, the fish was sustained in a prolonged induction phase of

aestivation which they described as “terrestrialization” (Wood et al., 2005b; Wilkie et al., 2007). “Terrestrialization” was achieved by spraying water onto the bottom of the container in which *P. dolloi* was induced to aestivate. Since the bottom of the container was wet, an incomplete cocoon was formed only along the dorsal-lateral cutaneous surface, with the ventral surface in direct and constant contact with water throughout the 5-month period. Unlike aestivating fish, those undergoing terrestrialization exhibited occasional movement and were not confronted with desiccation which should theoretically lead to tissue dehydration. Thus, it is unsurprising that Wilkie et al. (2007) and Staples et al. (2008) reported a substantial increase instead of a decrease in muscle water content in fish exposed to air for 5 months, and their results suggest that water was absorbed through the ventral cutaneous surface. During the initial phase of air exposure, water and ion fluxes in the experimental fish were comparable to those in fish kept in water. However, the water flux declined over time, indicating once again that adaptive changes could have occurred in the skin under such experimental conditions (Wilkie et al., 2007).

It is important to mention that observations made by Wilkie et al. (2007) and Staples et al. (2008) would not be manifested by fish during the maintenance phase of aestivation, whereby the ventral surface is encased completely in a dry mucus cocoon. In addition, if not because of the artificial extension of the induction phase to 5 month, tissue urea content would not have built up to high concentrations (13-fold). Without high levels of tissue urea, the magnitude of water retention in the muscle would have been dismal during an induction period of 6-8 days. On the contrary, a large intake of water is expected to occur in fish aroused from long term aestivation when water becomes available to the ventral body surface because of high concentrations of urea being accumulated in the body. At present, no information is available on changes in permeabilities of the skin to NH_3, water or ions in African lungfishes during the transition from the induction phase to the maintenance phase (when the body is encased completely in a dried mucus cocoon), and from the maintenance

phase to the arousal phase, of aestivation. Perhaps, efforts should be made in the future to examine such changes and to elucidate the mechanisms involved.

9.1.3. An increase in urea synthesis and a decrease in ammonia production

Results obtained from this study on *P. annectens* together with information available on other African lungfishes in the literature (Chew et al., 2004; Ip et al., 2005f) reveal that the two major mechanisms adopted by African lungfishes to deal with ammonia toxicity during emersion or aestivation are increased urea synthesis and decreased ammonia production. The traditional focus of nitrogen metabolism in aestivating lungfishes was on increased urea synthesis (Smith, 1930, 1935; Janssens, 1964; Janssens and Cohen, 1968a, b). Although decreased ammonia production was suspected to occur during aestivation (Janssens and Cohen, 1968a; Carlisky and Barrio, 1972), its importance during both the induction and the maintenance phases has not been confirmed until recently (Chew et al., 2003b, 2004; Loong et al., 2005; Ip et al., 2005f).

Chew et al. (2003b) demonstrated that there were significant increases in urea levels in the muscle (8-fold), liver (10.5-fold), and plasma (12.6-fold) of *P. dolloi* exposed to air for 6 days without entering into aestivation. There was also a significant increase in the urea excretion rate in fish exposed to air for 3 days or more. Taken together, these results indicate that *P. dolloi* increased the rate of urea synthesis to detoxify ammonia during this period. Aerial exposure also led to an increase in the hepatic ornithine-urea cycle capacity, with significant increases in the activities of CPS III (3.8-fold), argininosuccinate synthetase + lyase (1.8-fold) and glutamine synthetase (2.2-fold). In addition, the ammonia excretion rate in the experimental fish decreased significantly but there were no significant increases in ammonia contents in the muscle, liver or plasma, indicating that endogenous ammonia production was drastically reduced.

In this study (Chapter 1; Loong et al., 2005) reported that the rates of urea synthesis in *P. annectens* exposed to air for 6 days increased only 1.2- and 1.5-fold, respectively, which were smaller than that in *P. dolloi*. However, unlike *P. dolloi*, aerial exposure had no significant effects on the hepatic CPS III activities of *P. annectens*. Rather, aerial exposure induced relatively greater degrees of reductions in ammonia production in *P. annectens* (37%) compared with *P. dolloi* (28%). Thus, Loong et al. (2005) concluded that there were subtle differences in responses by various species of African lungfishes to aerial exposure, and it would appear that *P. annectens* depended more on a reduction in ammonia production than an increase in urea synthesis to ameliorate ammonia toxicity during the induction phase of aestivation. However, it is important to note that in Chapter 1, fish were prevented from aestivating by the daily renewal of a small amount of water at the bottom of the tank. It does not really reflect the physiology of the fish undergoing the induction phase of aestivation, during which the external medium was allowed to dry up within a 6-7 day period. Subsequently (Chapter 5), the author demonstrated that there were indeed up-regulations of mRNA expression of *cps*, *ass* and *gs* in *P. annectens*, especially during the induction phase (day 3 and day 6) indicating that there was indeed an increase in the OUC capacity, resulting in increased rate of urea synthesis and accumulation, during this period of aestivation.

In the case of *P. dolloi* exposed to air, the apparent decrease in ammonia production was associated with significant decreases in contents of glutamate, glutamine, lysine and total free amino acid in the liver (Chew et al., 2003b). Therefore, Chew et al. (2003b) interpreted that a decrease in proteolysis and amino acid catabolism could have occurred. However, the author holds a different view and believes that the reduction in ammonia production during the induction phase of aestivation should not be viewed as an adaptation responding solely to ammonia toxicity and conservation of metabolic fuels (Chew et al., 2003b). There could actually be an increase in protein synthesis, which would also result in decreases in ammonia production and in the total free amino acid content. Some animals (African lungfishes, Chew

et al., 2004; Loong et al., 2005, 2008b; green-striped burrowing frog, Booth, 2006) are known to secrete large quantities of mucus before aestivation. The mucus subsequently dries up to form a mucus cocoon which presumably functions to reduce evaporative water loss. The composition of the mucus cocoon is unclear at present, but there are indications that it comprises nitrogenous compounds (Bayomy et al., 2002). Therefore, it is logical to deduce that there could be an increase in the synthesis of certain proteins during the induction phase. Furthermore, structural changes have been identified recently as important facets of aestivation in African lungfishes (Icardo et al., 2008; Ojeda et al., 2008), and structural changes cannot occur without increased protein synthesis. Hence, results obtained by Chew et al. (2004) could be interpreted as the occurrence of increased protein synthesis and turnover instead of decreased protein degradation during the induction phase of aestivation. Since African lungfishes hyperventilate (Y. K. Ip and S. F. Chew, unpublished observations) during the initial period of the induction phase of aestivation, the reduction in ammonia production may not occur in association with metabolic depression, and there could be an increase in metabolic rate instead. Efforts should be made in the future to verify this hypothesis.

9.1.4. Molecular adaptation during the induction phase

Results obtained in this study (Chapter 4) revealed a number of genes expressed in liver of *P. annectens* during the induction phase of aestivation (i.e. 6 days of aestivation). Consistent with the findings in Chapter 3, aestivation in normoxia or hypoxia led to up-regulation of *cps*, *ass* and *gs*. There could also be an increase in protein turnover as indicated by both up- and down-regulation of certain genes related to translation of proteins. During the induction phase of aestivation, there was probably a need to modify protein strucures through increased protein synthesis and increased protein degradation in preparation of long-term aestivation. There could be increases in synthesis of certain types of haemoglobin

during induction phase. There were up-regulation of genes in iron and copper metabolism. There was a change in preference for lectin pathway in complement activation and suppression in blood clot formation. All the changes described above occurred in both aestivations in normoxia or in hypoxia. One interesting finding on these two conditions revealed that carbohydrate metabolism decreased in liver of fish aestivating in normoxia but increased in that of fish aestivating in hypoxia. This finding once again confirmed that differences existed aestivation in normoxia and aestivation in hypoxia. Hence, information available in the literature on aestivating lungfish should be reviewed with caution especially when there is no indication on the degree of hypoxia or its severity.

9.2. Nitrogen metabolism during the maintenance phase

9.2.1. Protein/amino acids as metabolic fuels versus preservation of muscle structure and strength

During aestivation, energy can be produced from the catabolism of lipids, ketone bodies, carbohydrates and/or proteins/amino acids (Fricks et al., 2008b), and the primary fuel preference varies between species. Large amounts of metabolic reserves, in the form of lipids and glycogen, accumulate in the prepupae of the arctiid moth, *Cymbalophora pudica*, prior to aestivation, and glycogen serves as the main metabolic fuel for aestivating prepupae (Kostal et al., 1998). Aestivating land snails (*Oreohelix strigosa* and *O. subrudis*) utilize predominately carbohydrates during the initial 2–4 months of aestivation, and protein is the primary metabolic substrate after polysaccharide reserves are depleted, with lipid being catabolized at a low rate throughout aestivation (Rees and Hand, 1993). For aestivating sea cucumber, *Apostichopus japonicus*, lipid and protein act as energy sources for large size specimens while protein is the major fuel for small size individuals (Yang et al., 2005, 2006). Many vertebrate species accumulate large glycogen stores prior to aestivation, but unlike hypoxic exposure, there is usually no instantaneous decrease in tissue glycogen contents

during the maintenance phase of aestivation. Hence, either glycogen degradation rate is extremely low, or glycogenolysis is compensated constantly by gluconeogenesis and glycogen synthesis. The continuous build up of urea in the body of aestivating African lungfishes suggests protein/amino acids as the major metabolic fuel during long-term aestivation (Janssen and Cohen, 1968a, b; Chew et al., 2004; Frick et al., 2008a), and the relatively unchanged levels of glycogen (Janssen and Cohen, 1968a, b; Frick et al., 2008a) indicates that a portion of the carbon skeletons derived from amino acid catabolism is channeled to gluconeogenesis. The yellow mud turtle, *Kinosternon flavescens*, has very high lipid content prior to aestivation (Long, 1985). Although catabolism of proteins and amino acids occur during aestivation, the rate of nitrogenous waste production is dependent on initial lipid stores (Peterson and Stone, 2000). Overall, it can be generalized that proteins/amino acids act as the major metabolic fuel in animals undergoing long-term aestivation. However, despite the metabolic demand for protein and amino acids, prominent increases in rates of proteolysis and amino acid catabolism do not occur in muscle of aestivators during the maintenance phase of aestivation.

Skeletal muscle atrophy refers to a loss of muscle volume and strength due to decreases in the size (hypotrophic) and/or the number (hypoplastic) of muscle fibers, resulting in a compromised capacity for physical work. Disused muscle atrophy occurs as a result of immobility (Childs, 2003). To date, disused muscle atrophy has only been examined in detail in one aestivator, i.e. the green-striped burrowing frog, *Cyclorana alboguttata* (Hudson and Franklin, 2002a, b, 2003; Hudson et al., 2006; Symonds et al., 2007). It has been reported that *C. alboguttata* can preserve muscle structure and function after 6-9 months of aestivation. At present, the actual mechanisms behind the preservation of muscle structure and function in aestivators and hibernators are unknown. For hibernating mammals, some interesting hypotheses have been raised, which include the positioning of the immobilized limb muscle in the over-wintering bears (Tinker et al., 1998) and the use of "shivering

thermogenesis" as an exercise cue in ground squirrels (Wickler et al., 1991). In addition, it has been hypothesized that hibernating bears can retain their skeletal muscle protein and strength by lowering the energy demand for proteolysis through metabolic rate reduction, drawing on labile protein reserves such as visceral smooth muscle and extracellular matrix, and/or synthesizing new amino acids and protein from urea nitrogen (Harlow et al., 2001). For the aestivating frog, *C. alboguttata*, Hudson et al. (2006) reported that transcriptional silencing of bioenergetic genes, such as NADH ubiquinone oxidoreductase 1, ATP synthase and superoxide dismutase 2 occurred in the skeletal muscle. They (Hudson et al., 2006) suggested that defenses against oxidative stress could be involved in the suppression of disuse muscle atrophy. Recently, Hudson et al. (2008) demonstrated that increases in expression of seven genes, which code for proteins with established roles in epigenetically-mediated gene silencing, occurred in the muscle of *C. alboguttata* after 6 months of aestivation, and therefore concluded that transcriptional silencing of skeletal muscle bioenergetic genes could involve chromatin remodeling. The mechanism of how African lungfish is able to swim and feed after prolonged period of aestivation is unknown. Hence, they provide an excellent model for the study of skeletal muscle disuse.

9.2.2. Reduction in ammonia production and changes in hepatic GDH activity

Chew et al. (2004) demonstrated that the rate of ammonia production in the African lungfish *P. dolloi* reduced to 26% and 28% during the first 6 and the subsequent 34 days of aestivation, respectively, as compared with the day 0 control value of 6.83 μmol day^{-1} g^{-1}. For *P. aethiopicus* that underwent 46 days of aestivation, there was only a 20% decrease in the rate of ammonia production during the initial 12 days, but a profound decrease (96%) in ammonia production occurred during the final 12 days of aestivation (day 34 to day 46) (Ip et al., 2005f). Hence, different African lungfish species exhibit different capacity of reduction in ammonia production.

In this study, the author examined the importance of increased urea synthesis and decreased ammonia production in *P. annectens* aestivating in air versus those aestivating in mud (Chapter 2; Loong et al., 2008b). Twelve days of aestivation in air led to significant increases in contents of urea, but not ammonia, in tissues of *P. annectens*. The estimated rate of urea synthesis increased 2.7-fold despite the lack of changes in the activities of hepatic ornithine-urea cycle enzymes, but there was only a minor change in the estimated rate of ammonia production. After 46 days of aestivation in air, the ammonia content in the liver decreased significantly and content of urea in all tissues studied increased significantly, indicating that the fish shifted to a combination of increased urea synthesis (1.4-fold of the day 0 value) and decreased ammonia production (56% of the day 0 value) to defend against ammonia toxicity. By contrast, 12 days of aestivation in mud produced only minor increases in tissue urea content, with ammonia content remained unchanged. This was apparently achieved through decreases in urea synthesis and ammonia production (40% and 15%, respectively, of the corresponding day 0 value). Surprisingly, 46 days of aestivation in mud resulted in no changes in tissue urea content, indicating that profound suppressions of urea synthesis and ammonia production (2.6% and 1.2%, respectively, of the corresponding day 0 value) had occurred. Since fish aestivated in mud had relatively low blood pO_2 and muscle ATP content, they could have been exposed to hypoxia, which induced reductions in metabolic rate and ammonia production. Consequently, fish aestivating in mud had a lower dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, than fish aestivating in air.

Since transdeamination is an important pathway of amino acid catabolism, GDH is in a crucial position to regulate ammonia production (see Section 6.1). Janssens and Cohen (1968b) suspected but without supportive data that reduction in ammonia production in aestivating *P. aethiopicus* was achieved through the regulation of GDH activities. GDH is known to be activated by ADP (Campbell, 1973), the concentration of which may change

during hypoxic exposure, and GDH can also be modified by ADP-ribosylation (Herrero-Yraola et al., 2001). In this study (Chapter 3; Loong et al., 2008a), the author examined whether there would be changes in specific activity and kinetic properties of GDH from the liver of *P. annectens* during the induction and maintenance phases of aestivation, and whether these changes would be different between normoxic and hypoxic fishes, especially with regard to ADP activation *in vitro*. It was discovered that the activities of hepatic GDH, in the amination and deamination directions, remained relatively constant in fish exposed to normoxia during the induction phase (3 or 6 days) of aestivation (Chapter 3; Loong et al., 2008a). However, there was a significant increase in the GDH amination activity, with the deamination activity remained unchanged, in fish aestivating in normoxia on day 12. Hence, GDH would act less favourably in the deamination direction during the maintenance phase of aestivation to reduce the production of ammonia through transdeamination. Simultaneously, the hepatic GDH amination activity, but not the deamination activity, from fish aestivating in normoxia on day 12 became highly dependent on the presence of ADP. These results indicate that transdeamination of amino acids through the hepatic GDH became responsive mainly to the cellular energy status of the fish during the maintenance phase of aestivation (day 12) in normoxia. Since ammonia concentrations in various tissues of *P. annectens* exposed to normoxia (or hypoxia) remained relatively unchanged (Chapter 3; Loong et al., 2008a), it can be concluded that changes in the activity of hepatic GDH occurred primarily to reduce ammonia production, and not to detoxify ammonia during aestivation. In comparison, for fish exposed to hypoxia, significant increases in the hepatic GDH amination activity, the amination/deamination ratio and the dependency of the amination activity on ADP activation occurred much earlier on day 6, i.e. at the onset of aestivation, instead of day 12 (Chapter 3; Loong et al., 2008a). These results indicate that decreased ammonia production through changes in the activity of hepatic GDH in *P. annectens* could be more effectively induced and exacerbated by a combination of aestivation and hypoxia than aestivation alone (in

normoxia). In addition, they suggest that GDH was critically regulated in fish during the transition between the induction and the maintenance phases of aestivation in hypoxia, suppressing ammonia production in order to reduce the dependency on increased urea synthesis to detoxify ammonia.

Results reported in Chapter 5 reveal that the regulation of GDH in the liver of *P. annectens* during the induction and early maintenance phase of aestivation in normoxia or hypoxia was not involve in the increase of mRNA expression of the GDH gene. It is therefore probable that GDH was regulated at the post-transcriptional and post-translational level, possibly through covalent modification.

9.2.3. Changes in the rate of urea synthesis and activities of ornithine-urea cycle enzymes

In spite of suppressing ammonia production during the maintenance phase of aestivation, endogenous ammonia must be detoxified because its excretion would have been completely impeded during long periods of desiccation. In many cases, ammonia is detoxified to urea through the hepatic ornithine-urea cycle. By synthesizing and accumulating the moderately less toxic urea, animals can carry out protein catabolism for a longer period without being intoxicated by ammonia. Chew et al. (2004) reported that the urea synthesis rates of *P. dolloi* increased 2.4-fold and 3.8-fold during the first 6 and the subsequent 34 days of aestivation, respectively, compared with the day 0 control value; and urea accumulated in various tissues of fish aestivated for 6 or 40 days. Although activities of ornithine-urea cycle enzymes in fish aestivated for 6 days remained unchanged, the activities of several ornithine-urea cycle enzymes increased significantly in fish aestivated for 40 days.

Previous works by Janssens and Cohen (1968a) also showed that urea accumulation occurred in *P. aethiopicus* aestivated for 78-129 days in an artificial mud cocoon. However, by injecting ^{14}C-bicarbonate into fish aestivated for 78-129 days and quantifying radio-labeled urea during a subsequent 60-h period, Janssens and Cohen (1968a) concluded that

urea accumulation in *P. aethiopicus* did not involve an increase in the rate of urea synthesis, even though the fish appeared to be in continuous gluconeogenesis throughout aestivation. Subsequently, Ip et al. (2005f) undertook a study to test the hypothesis that the urea synthesis rate in *P. aethiopicus* was up-regulated to detoxify ammonia during the initial period of aestivation (day 0 to day 12), and that a profound suppression of ammonia production occurred at a later period of aestivation (day 34 to day 46) which eliminated the need to sustain the increased rate of urea synthesis. Contrary to the report of Jannsens and Cohen (1968a), results of Ip et al. (2005f) demonstrated a drastic increase in urea synthesis (3-fold) in *P. aethiopicus* during the initial 12 days of aestivation, although the magnitude of the increase in urea synthesis decreased over the next 34 days. Between day 34 and day 46 (12 days), the rate of urea synthesis decreased to 42% of the day 0 control value instead. There were significant increases in tissue urea content and activities of some ornithine-urea cycle enzymes from the liver (Ip et al., 2005f). Since there was a meager 20% decrease in the rate of ammonia production in *P. aethiopicus* during the initial 12 days, as compared to a 96% decrease during the final 12 days of aestivation (day 34 to day 46), Ip et al. (2005f) concluded that *P. aethiopicus* depended mainly on increased urea synthesis to ameliorate ammonia toxicity during the initial period of aestivation, but during prolonged aestivation, it suppressed ammonia production profoundly, eliminating the need to increase urea synthesis which is energy intensive.

In this study (Chapter 3; Loong et al., 2008a), the author also reported that *P. annectens* exhibited different adaptive responses during aestivation in normoxia and in hypoxia. Ammonia toxicity was avoided by increased urea synthesis and/or decreased endogenous ammonia production, but the dependency on these two mechanisms differed between the normoxic and the hypoxic fish. The rate of urea synthesis increased 2.4-fold, with only a 12% decrease in the rate of ammonia production in the normoxic fish. In contrast, the rate of ammonia production in the hypoxic fish decreased by 58%, with no

increase in the rate of urea synthesis. Using *in vivo* ^{31}P NMR spectroscopy, it was demonstrated that hypoxia led to significantly lower ATP concentration on day 12 and significantly lower creatine phosphate concentration on days 1, 6, 9 and 12 in the anterior region of the fish as compared with normoxia. Additionally, the hypoxic fish had lower creatine phosphate concentration in the middle region than the normoxic fish on day 9. Hence, lowering the dependency on increased urea synthesis to detoxify ammonia by reducing ammonia production would conserve cellular energy during aestivation in hypoxia.

The changes in hepatic OUC capacity in *P. annectens* during the induction and maintenance phases of aestivation apparently involved the regulation of mRNA expression of *cps*, presumably *cps* III, and *ass*, with *cps* as the major regulatory enzyme (Chapter 5). Indeed, aestivation in normoxia or hypoxia exerted different effects on *cps* and *ass* mRNA expression.

9.2.4. Levels of accumulated urea and mortality

Rees and Hand (1993) studied biochemical changes occurring over 7 months of aestivation in two species of land snails, *O. strigosa* and *O. subrudis*, to determine whether differential mortality during aestivation was related to different energetic strategies. Laboratory-maintained snails, which were fed *ad libitum* prior to aestivation, were compared with snails collected from the field and induced to aestivate without augmenting their energy reserves. If the duration of aestivation was limited by the depletion of energy storage compounds during aestivation, then snails with larger stores prior to aestivation would be predicted to survive aestivation proportionately longer. Indeed, snails with elevated level of polysaccharide had lower mortality during aestivation as compared to snails collected from the field, and the negative correlation between pre-aestivation polysaccharide stores and mortality was statistically significant. However, the observation that polysaccharide stores were exhausted several months prior to the onset of mortality suggests that mortality was not

due to the depletion of this substrate. On the other hand, the tissue urea concentrations (150-300 mM) were positively correlated with mortality in these snails. Since methylamine compounds that can offset disruptive effects of elevated urea, were present in low concentrations, Rees and Hand (1993) suggested that in the absence of elevated levels of counteracting compounds, urea might reach toxic levels and might be one factor limiting the duration of aestivation without mortality. If urea does reach toxic levels, then it raises the question: why do aestivating snails and other aestivators like *P. annectens* (and other African lungfishes) synthesize and accumulate urea?

9.2.5. Accumulation of urea—Why?

Many aestivators accumulate urea in their body fluids, and urea concentrations can often reach several hundred millimolar (Jones, 1980; Grundy and Storey, 1998). Urea is well known for its ability to denature proteins and exerts disruptive effects on enzymes, (Hochachka and Somero, 1984), and there is little evidence of the presence of methylamines or other potential counteracting solutes in aestivating animals that accumulate urea (McClanahan, 1967; Withers and Guppy, 1996). Fuery et al. (1997) proposed that an adaptation for tolerance of high urea existed in the lactate dehydrogenase of the aestivating Australian desert frog as compared with those of non-aestivating species. On the other hand, it has been suggested that accumulated urea contributes to metabolic depression in dormant animals (Griffith, 1991) by reversibly inhibiting key metabolic enzymes (Hand and Somero, 1982; Yancey et al., 1982). Indeed, recent studies (Costanzo and Lee, 2005; Muir et al., 2007) on hibernating wood frogs (*Rana sylvatica*) suggest a link between urea accumulation and metabolic depression. Muir et al. (2008) measured aerobic metabolism of isolated organs from the wood frog in the presence or absence of elevated urea at various temperatures using frogs acclimatized to different seasons. When organs from winter frogs were tested at 10^{o}C, metabolism was significantly decreased in urea-treated liver and stomach by ~15% and in

urea-treated skeletal muscle by ~50%. Therefore, Muir et al. (2008) concluded that the presence of urea depressed the metabolism of living organs, and thereby reduced energy expenditure.

Grundy and Storey (1994, 1998) analyzed the effects of urea on selected enzymes involved in intermediary metabolism and antioxidant defense in spadefoot toad organs and compared these with the effects of KCl. Urea (200 mM) had no effect on pyruvate kinase, phosphofructose kinase 1 and isocitrate dehydrogenase but lowered GDH activity to 65% of control values. By contrast, 200 mM KCl inhibited all four enzymes with a particularly strong effect on GDH activity, indicating that spadefoot toad enzymes are much more sensitive to high KCl concentrations than to high urea concentrations. Therefore, it would appear that urea accumulation minimizes the elevation of cellular ionic strength that would otherwise occur and affect enzyme activities (Grundy and Storey, 1998; Cowen and Storey, 2002).

Riddle (1983) proposed that urea accumulated during the maintenance phase of aestivation could facilitate water uptake from the environment upon re-hydration during arousal. In addition, there is also evidence which suggests that urea accumulation is essential to hydration status of tissues during aestivation. Arad (2001) studied the physiological responses to combinations of desiccation and rehydration in a Mediterranean land snail, *Theba pisana*. Their results indicated that urea was transported from the pallial fluid to the soft body tissue, facilitating water movement to and maintaining the hydration status of the latter. On the other hand, the burrowing frog, *Cyclorana australis*, digs shallow burrows while the soils are still quite moist at the beginning of the dry season. It spends 2-3 months underground without cocoons, and cocoon formation occurs only when the soils dry to an extent that would dehydrate the frogs. During the first part of aestivation before the formation of cocoon, it absorbs water from the environment (Booth, 2006; Tracy et al., 2007), probably as a result of accumulation of urea in tissues and body fluids. Increased urea levels in tissues

also facilitate the mobilization of water stored in the urinary bladder during the later phase of aestivation (Cartledge et al., 2008).

It has been previously suggested that aestivation in air entails desiccation and increased tissue urea contents might serve the secondary function of facilitating water retention in tissues through vapour pressure depression (Horne, 1971; Campbell, 1973; Land and Bernier, 1995; Withers and Guppy, 1996; Withers, 1998; Storey, 2002). However, this proposition may be invalid for several reasons. First, it has been established that urea concentration of 300 mM has only minor contribution to the gradient for water movement between tissues and dry air (Machin, 1975). Secondly, contrary to the suggestions that urea may have an osmotic role in aestivating snails (Campbell, 1973; Horne, 1971; Bishop et al., 1983), Horne (1973a) demonstrated that *B. dealbatus* which underwent aestivation in a 85% relative humidity remained active for a longer period before aestivation and accumulated urea at a faster rate than those aestivated in 14% relative humidity (Horne, 1973a). As a result, Horne (1973a, b) concluded that increases in urea synthesis and accumulation in aestivating *B. dealbatus* were unrelated to water conservation, but occurred as a result of ammonia detoxification in conjunction with an increase in protein degradation during fasting. Working on the giant African snail, *A. fulica*, Hiong et al. (2005) came to the same conclusion. *A. fulica* accumulated urea progressively not only during 23 days of aestivation, but also during 23 days of fasting (Hiong et al., 2005). Since *A. fulica* was fasted in an ample supply of water and since fasting had no significant effects on the water contents of the foot muscle and hepatopancreas, urea accumulation did not occur because of the need to retain water through a decrease in the partial pressure of water vapor. Thirdly, in this study (Chapter 3; Loong et al., 2008a), the author experimented on two groups of *P. annectens* that underwent aestivation in closed boxes with similar flow rates of air or 2% O_2 in N_2, and hence experienced similar magnitudes of desiccation. However, fish aestivating in hypoxia suppressed ammonia production and consequently accumulated much less urea. Therefore, it

can be concluded that increased urea synthesis in *P. annectens* (and probably other African lungfishes) is an adaptation responding primarily to rates of protein degradation and amino acid catabolism (Chapter 3; Loong et al., 2008a).

9.3. Nitrogen metabolism and excretion during arousal from aestivation

9.3.1. Rehydration

The environmental cues that signal animals to arouse from aestivation have not been explicitly defined but water availability can naturally be regarded as one of the most important factors. In our laboratories, we routinely arouse African lungfish from aestivation in air by re-immersion. It has been suggested that the burrowing frog, *C. australis*, emerges from soils when the osmoticity of soil water becomes low enough to permit water absorption (Tracy et al., 2007).

Although Wilkie et al. (2007) studied *P. dolloi* during prolonged (5 months) exposure to air, their results actually offered insights into what would happen during the arousal phase when water becomes once again available to aestivating African lungfishes. Their results (Wilkie et al., 2007) indirectly support the proposition of Riddle (1983), because the 13-fold increase in muscle urea content was the likely explanation for the 56% increase in muscle water content observed after 5 months of air exposure. However, the phenomena reported by Wilkie et al. (2007) that muscle acted as a "water reservoir" during air exposure and that the body mass decreased by 20% during subsequent re-immersion in water might not reflect the real situation of arousal from aestivation. Firstly, the level of urea accumulated in the body during the normally short period of induction phase of aestivation would not lead to such a large increase in muscle water content. Secondly, after a long period of aestivation, there should be a decrease, and not an increase, in the muscle water content. Thirdly, during arousal, it is essential for the fish to gain water from instead of losing it to the environment. Although it is unlikely that fish can accumulate such high levels of urea during a 6-8 day

induction period, similar magnitude of increase in urea content can be expected to have occurred in fish that underwent an equivalent period (i.e. 5 months) of aestivation. Therefore, it can be deduced from results reported by Wilkie et al. (2007) that water absorption occurs through the ventral body surface of African lungfish as water becomes available during arousal.

This offers insight in part into the reason for the accumulation of urea instead of uric acid (or other purines) during the maintenance phase of aestivation; urea being water soluble is osmotically active while uric acid is largely insoluble in water. Following this line of deduction, water absorption must precede urea excretion because urea is crucial to this osmotic phenomenon during arousal.

9.3.2. Excretion of accumulated urea

Urea accumulated in the body of aestivating lungfishes can be excreted effectively during arousal in water (Smith, 1930; Janssens, 1964). Chew et al. (2003b) working on *P. dolloi* exposed to terrestrial conditions for 6 days demonstrated that the urea excretion rate increased 22-fold during re-immersion as compared to the control specimen. This is the greatest increase in urea excretion amongst fishes during emersion-immersion transition, and suggests that *P. dolloi* possesses transporters which facilitate the excretion of urea in water. Subsequently, Wood et al. (2005b) reported that after 21-30 days of aestivation in air or exposed to air without aestivation, the urea excretion rate was greatly elevated in *P. dolloi* during re-immersion, reaching 2000-6000 μmol-N h^{-1} kg^{-1} at 10–24 h after return to water. A divided chamber experiment demonstrated that 72% of the urea-N efflux occurred through the posterior 85% of the body, with minimal involvement of the kidney, thereby pointing to the skin as an important site of urea-N excretion. Wood et al. (2005b) discovered that urea was excreted by *P. dolloi* in pulses during re-immersion but the reason behind such a phenomenon was unclear. Perhaps, it is an adaptation to assure complete rehydration, which

is dependent on tissue urea content, upon arousal. Through the injection of NH_4Cl + urea, Ip et al. (2005d) concluded that excretion of accumulated urea in *P. dolloi* was regulated by the level of internal ammonia in its body. It is probable that there is an increase in ammonia production through increased amino acid catabolism upon arousal, and the increased production of endogenous ammonia could act as a signal to increase urea excretion.

Recently, the full length cDNA sequence of a putative urea transporter (lfUT) of the UT-A type has been cloned from *P. annectens* (Hung et al., 2009). The lfUT cDNA is 1990 bp in length and its open reading frame encodes a 409 amino acid long protein, with a calculated molecular weight of 44,723 Da. The sequence is closest to those of amphibians (~65 % amino acid homology), followed by mammals and elasmobranchs (~60 %), and then teleosts (~50 %). lfUT was clearly expressed in gill, kidney, liver, skeletal muscle, and skin. Upon re-immersion in water after 33 days of air exposure, *P. annectens* exhibited a massive rise in urea-N excretion which peaked at 12–30 h with rates of 2000–5000 μmol N kg^{-1} h^{-1} and persisted until 70 h. Quantitative RT-PCR revealed significant elevation of lfUT expression in the skin between hour 14 and 48 of re-immersion. Thus, it can be deduced that transcriptional activation of lfUT would occur in the skin of African lungfishes to facilitate urea excretion during the arousal phase of aestivation.

9.3.3. Feeding, tissue regeneration and protein synthesis

The increased excretion of urea during re-hydration and changes in osmolality of tissue fluids may be involved indirectly in tissue regeneration upon arousal from aestivation. Working on *P. dolloi* during the maintenance and arousal phases of aestivation, Icardo et al. (2008) reported that the heart had high capacity for functional recovery. They proposed that the drastic reduction in the amounts of urea accumulated in the body tissues that occurs upon arousal (Wood et al. 2005b) may produce an osmotic imbalance that eventually results in rupture of the membranes and the massive accumulation of the vacuolized cytoplasm

components in the septal myocytes of the heart. Subsequently, these areas attract the macrophages involved in debris clearance, and such a process may facilitate tissue regeneration.

During aestivation, the intestine of the green-stripped burrowing frog, *C. alboguttata*, undergoes significant morphological down-regulation, but there is rapid restoration of intestinal morphology upon arousal from aestivation and during the initial stages of re-feeding (Cramp and Franklin, 2005; Cramp et al., 2005). Arousal alone has a marked impact on many morphological parameters, including small and large intestine masses, small intestinal length, enterocyte cross-sectional area and microvilli height and density (Cramp and Franklin, 2005). Such structural changes would require increased syntheses of certain proteins, and since they occur before re-feeding, it would imply the mobilization of amino acids of endogenous origin. Upon feeding, *C. alboguttata* employs reduced digesta passage rates so as to maximize nutrient assimilation efficiency following prolonged food deprivation during aestivation (Cramp and Franklin, 2003). However, at present, there is a dearth of knowledge on protein degradation and synthesis during the arousal of aestivating animals. Furthermore, no information is available on postprandial nitrogen metabolism and excretion in animals upon arousal from aestivation. These findings hinted to us that the cell death and cell proliferation were occurring in different tissues and at different phase of aestivation in the African lungfish. Understanding cell death and proliferation can add on to our understanding on tumor growth and cancer formation.

9.3.4. Important roles of GDH and GS during arousal

Results obtained in this study (Chapter 5) revealed for the first time hepatic GDH and GS might have important physiological roles in *P. annectens* during arousal as their mRNA expression were up-regulated either before or right after arousal from aestivation. Such a role of GDH and GS has never been explored or examined for aestivating animals and hence no

information is available in the literature in this regard for a meaningful discussion at present. Effort should be made in the future to elucidate the function of GDH and GS not only after arousal from aestivation but also after the initiation of feeding 7-10 days arousal. The author surmises that there would be major postprandial changes in the activities, kinetic properties, mRNA and protein expression, and perhaps even isoforms, of these two enzymes in the liver of fish upon arousal from aestivation.

9.4. Summary

Based on results obtained from *P. annectens* in this study and those reported in the literature, it can be concluded that adaptive responses exhibited by aestivators with regard to excretory nitrogen metabolism during the induction and the maintenance phases, and perhaps also the arousal phase, of aestivation differ from those exhibited by non-aestivators undergoing fasting or immobilization, although aestivation involves long-term fasting and corporal torpor. At present, not much information is available on excretory nitrogen metabolism in animals, including African lungfishes, during the initial phase of or upon arousal from aestivation. Therefore, future efforts should be made to identify adaptive responses particular to each of the three phases of aestivation. Since structural changes are expected to occur during the induction and arousal phases, it would be essential to study the intricate relationship between protein synthesis and protein degradation and the resulting rapid turnover of nitrogen in certain organs of animals going through these two phases of aestivation. In addition, efforts should be made in the future to study mechanisms involved in the suppression of protein degradation to preserve proteinaeous structures and the regulation of GDH to reduce ammonia production. As for nitrogenous products, it would be important to further elucidate the functional roles of urea, and also other nitrogen containing compounds, during the induction and maintenance phases, and to examine how urea excretion is regulated to facilitate complete rehydration upon arousal.

10. References

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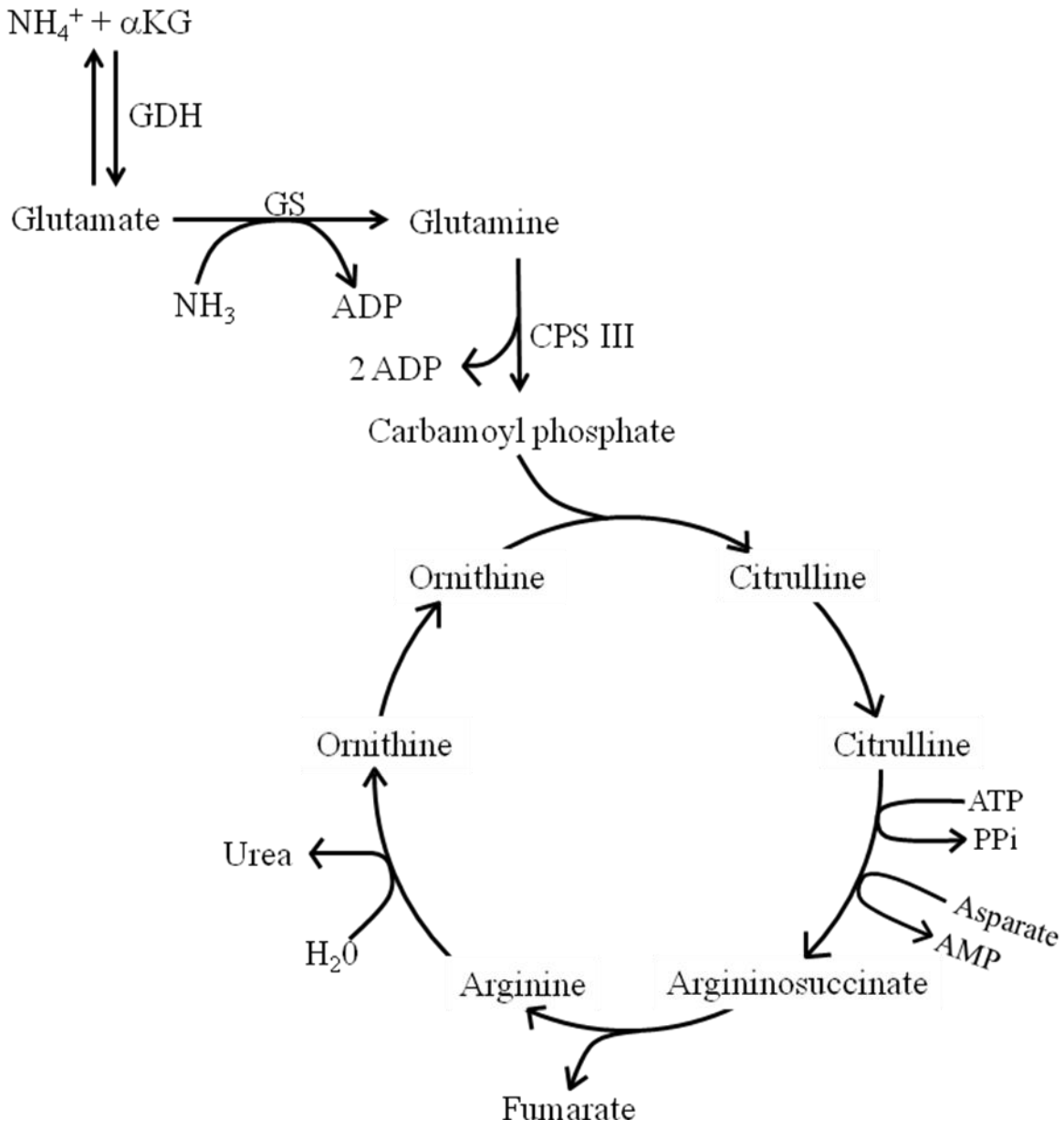
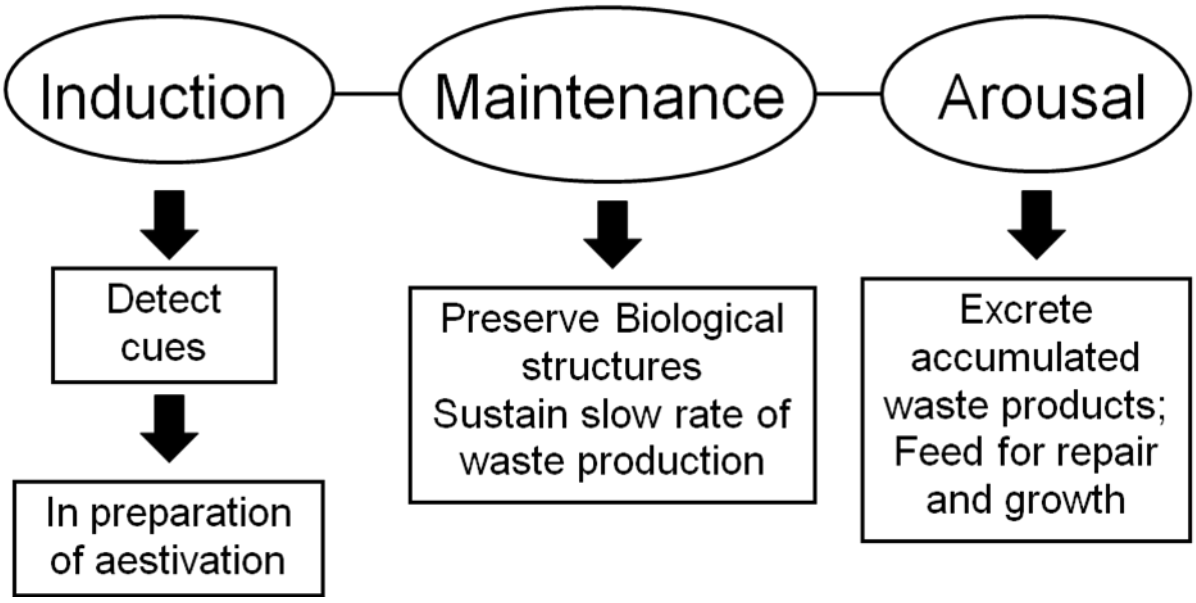
Appendix 1.


NH4+ + αKG
GDH
Glutamate
GS
Glutamine
NH3
ADP
CPS III
2 ADP
Carbamoyl phosphate
Ornithine
Citrulline
Ornithine
Citrulline
ATP
PPi
Urea
Asparate
AMP
H20
Arginine
Argininosuccinate
Fumarate

Appendix 2.


Induction
Maintenance
Arousal
Detect cues
In preparation of aestivation
Preserve Biological structures
Sustain slow rate of waste production
Excrete accumulated waste products; Feed for repair and growth